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Development of solid phase extractants for pesticide analysis

By

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I, Paul Josef Myburgh, declare that the dissertation, which I hereby submit for the degree MSc Chemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

Date: 2020/12/14

Summary

Solid phase extraction (SPE) is a well-known sample preparation technique which is used widely by many ISO 17025 accredited laboratories around the world and is also the focus of many articles in the academic literature. Furthermore, with regards to the use of SPE sorbents, molecularly imprinted polymers (MIPs) have received significant research interest due to the need for selective and precise extraction sorbents.

The four pesticide templates used in this study were selected specifically for their relevance in the South African context, namely two triazine pesticides: atrazine and terbuthylazine, and two chloroacetanilide class pesticides: acetochlor and alachlor. All four of the selected pesticides are highly ranked locally in terms of their weighted hazard potential based on the quantity used, their toxicity and potential environmental impacts. Another factor that played an important role in the pesticide selection process was compatibility with gas chromatography-mass spectrometry (GC-MS) analysis. Even though liquid chromatography tandem mass spectrometry (LC-MS/MS) is a powerful, more sensitive alternative, it is not readily available in many commercial laboratories due to its high cost. More analyte concentration and sample clean-up steps were thus necessary prior to sample analysis by GC-MS. In this study, emphasis was additionally placed on pesticides that have the potential to be used in the South African medicinal cannabis field. As of 2017, there has been a heightened interest in cannabis as South Africa started moving towards the legalisation thereof for personal and medicinal use.

In this study, MIPs and non-imprinted polymers (NIPs) were synthesised utilising methacrylic acid (MAA) as functional monomer, ethylene glycol dimethacrylate (EGDMA) as cross-linker and 2,2'-azobis (2-methylpropionitrile) (AIBN) as a radical polymerization activator. Several MIPs were synthesised, including a multi-template MIP, which included all four target pesticide templates in one MIP. The template molecules were removed by repeated washing of the polymer with a mixture of methanol: acetic acid (9:1 v/v). Size fractionation was performed utilising wet sieving, with 25 and 53 µm stainless steel sieves and deionized water. The washed and size-fractionated polymers were subsequently packed into cartridges suitable for SPE. The materials were characterized by scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR).

The adsorption capacities were determined for each synthesised MIP, as well as the NIP. The NIP adsorption capacity ranged from 0.52 to 0.69 mg/g for atrazine and alachlor, respectively. Comparing the average adsorption capacity of the two pesticide classes on the NIP indicates that the chloroacetanilide pesticides (namely acetochlor and alachlor) have higher adsorption capacities. A correlation between log K_{ow} values of the pesticides and adsorption capacities was observed.

Furthermore, the average MIP was found to have an adsorption capacity of 0.99 mg/g, with no significant statistical differences observed between the pesticide classes. The correlation between adsorption capacity and log K_{ow} was not observed as was the case in the NIP, indicating that more than just hydrophobic interactions were responsible for the increased adsorption capacity in the MIP. The MIP has adsorption sites or cavities left in the shape of the template molecule allowing for greater adsorption capacity when compared to the NIP. Several variations of MIPs and their corresponding NIPs were synthesised and characterized in terms of adsorption capacity and selectivity. The variations included increasing the amount of template added during synthesis, as it was hypothesised that an increase in the number of template molecules should increase the number of cavities in the subsequent MIP, thereby enhancing the adsorption capacity, and these MIPs were referred to as enhanced adsorption capacity (EAC) MIPs. A novel multi template MIP was also synthesised where both triazine and chloroacetamide pesticide class template molecules were added during synthesis.

Packed molecularly imprinted SPE (MISPE) cartridges were compared to commercially available C18 SPE cartridges in terms of extraction and elution efficiency. Under ideal conditions and a relatively high concentration of all four selected pesticides in the loading fractions (0.2 μg/mL), recoveries ranged from 90 to 97% for both the MISPE and C18 sorbents. Furthermore, it was found that the pesticides eluted more easily from the C18 sorbent than from their respective MIPs, as more methanol elution solvent had to be passed through the MIP to fully elute the analytes. Thus the cavities in the MIP provide high affinity adsorption sites for the template pesticide, making elution thereof more difficult.

Cannabis flower samples were spiked with the four selected pesticides, at the relevant concentration of 0.05 mg/kg, which is the South African maximum residue limit (MRL) for the selected pesticides on crops. The spiked flowers were then extracted utilising the MISPEs to good effect. With the spiked samples, the MIP outcompeted the C18 sorbent in terms of selectivity at the South African limit, as many non-polar molecules (oils and waxes found in cannabis plant material) were trapped on the C18 sorbent but can more easily pass through the selective synthesised MISPEs during the washing steps, resulting in less background interference. Pesticide recoveries from the MIP ranged between 58.5% for atrazine on an atrazine MIP with water extraction, to 85% recovery of acetochlor on the acetochlor MISPE with water extraction. It is theorised that the triazine pesticides have more sites for hydrogen bonding with MAA and EDGMA in the molecular cavities, as the NIP did not perform well for pesticide extraction from plant samples spiked at 0.05 mg/kg as no pesticides were detected in the NISPE extract.

In conclusion, the synthesised MIPs were effective at extracting pesticides from spiked cannabis material. The triazine MIPs proved to be slightly more efficient than the chloroacetamide MIPs as more background can be removed during the MISPE procedure, as observed by the number of matrix interference peaks present in the resulting chromatograms. This is attributed to the wash solvent, as atrazine and terbuthylazine loaded on the triazine MIPs are amenable to higher concentration methanol solvent fractions before the pesticide analytes are washed out of the cavities. Atrazine had the best recovery at 78.6% on the atrazine MISPE and acetochlor had the best recovery on the acetochlor MISPE at 79.1%, these recoveries were not found to be statistically different from one another with a t-test. For the chloroacetamide MIPs, it was found that the analytes were removed from the cavities far more easily, requiring the use of more polar loading and washing fractions. The enhanced adsorption capacity (EAC) MIP proved to have higher adsorption capacities for both the triazine and chloroacetamide pesticide classes. For triazine EAC MIPs the average adsorption capacity was increased from 0.93 to 1.32 mg/g for the triazine pesticides. Similarly, the adsorption capacity for the chloroacetamide pesticides increased from 1.02 to 1.24 mg/g on the chloroacetamide EAC MIPs. Thus proving the hypothesis that more cavities in the MIP increases the mass of analytes that can be adsorbed. It was, however, concluded that at a relevant spiking concentration of 0.05 mg/kg the increased adsorption capacity becomes irrelevant as only a few μg of pesticide analyte would be available for extraction prior to analysis. The novel multi template (MT) MIP proved to be effective at extracting and adsorbing all the targeted analytes from an aqueous solution and spiked cannabis flowers respectively, with recoveries ranging from 76.5% for atrazine and 83.2% for acetochlor with optimized extraction methods for each analyte.

In comparison with commercial C18 cartridges, the MISPEs performed better in terms of selectivity when spiked cannabis samples were analysed resulting in less background noise. However, the MIPs were found to be much more prone to channelling when the sorbent bed dried, which made it necessary to omit the drying step before the final elution of the analytes, which contributed to the increase of the elution fraction volume. The larger elution volumes do not, however, have a significant impact on the extraction recoveries, as they are dried and reconstituted with methanol containing internal standard prior to GC-MS analysis.

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List of Abbreviations

General abbreviations	
Abbreviation	**Description**
AC	Adaxial cuticle
AE	Adaxial epidermal
ATR	Attenuated total reflectance
BC	Basal cuticula
BE	Basal epidermal
COA	Certificate of analysis
CV	Conventional solvent
DAFF	South African Department of Agriculture, Forestry and Fisheries
dSPE	Dispersive solid phase extraction
EAC MIP	Enhanced adsorption capacity molecularly imprinted polymer
EI	Electron ionization
EPA	Environmental Protection Agency
FECO	Full extract cannabis oil
FID	Flame ionization detector
FTIR	Fourier-transform infrared
GC	Gas chromatography
GT	Glandular trichomes
HPLC	High performance liquid chromatography
IR	Infra-red
IS	Internal standard
LC	Liquid chromatography
LD	Lethal dose
LOD	Limit of detection
LOQ	Limit of quantification
LLE	Liquid-liquid extraction
MAE	Microwave-assisted extraction
Min	Minute
MIP	Molecularly imprinted polymer
MISPE	Molecularly imprinted solid phase extraction
MCX®	Mixed-mode cation exchange
MRL	Maximum residue limit
MS	Mass spectrometry
MTMIP	Multi-template molecularly imprinted polymer
NAFS	National Analytical Forensic Services
ND	Not detected
NGT	Non- glandular trichomes
NIP	Non-imprinted polymer
NISPE	Non-imprinted solid-phase extraction
PIPs	Plant incorporated protectants
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
Rpm	Revolutions per minute
RSD	Relative standard deviation
SAPS	South African Police Service
SAHPRA	South African Health Products Regulatory Authority
SAX	Strong anion exchange

General abbreviations	
Abbreviation	**Description**
SEM	Scanning electron microscopy
SIR	Selected ion recording
SIM	Selected ion monitoring
S/N	Signal to noise ratio
SPE	Solid-phase extraction
SPME	Solid phase micro extraction
STDEV	Standard deviation
TIC	Total ion current
UAE	Ultrasound-assisted extraction
UHPLC	Ultra High Performance Liquid Chromatography
UV	Ultraviolet
WHP	Weighted hazard potential
WS	Working standard

Chemical abbreviations	
Abbreviation	**Full name**
AIBN	2,2’ -Azobisisobutyronitrile
BETX	Benzene, toluene, ethylbenzene, and xylene
C18	Octadecylsilyl
CBD	Cannabidiol
DMDCS	Dimethylchlorosilane
DVB	Divinylbenzene
EDGMA	Ethylene glycoldimethacrylate
EtOH	Ethanol
MAA	Methacrylic acid
MBAAM	N,N-methylene bisacrylamide
MEHQ	4-Methoxyphenol
PFTBA	Perfluorotributylamine
PSA	Primary-secondary amine
PTFE	Polytetrafluoroethylene
THC	Tetrahydrocannabinol

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Chapter 1 : Introduction

1.1: Problem statement and rationale

Contamination can happen during any step of the analytical procedure, from sampling and sample preparation to the actual analysis, which can be problematic for low level analysis. Trace analysis is also hampered by the matrix background (or noise) and other interferences. High noise or background diminishes the analyte signal to noise ratio, leading to elevated limits of detection (LODs) and potentially inaccurate results. The removal of the background and contamination is thus of importance for trace analysis. The background and interferences present are determined by the sample itself, glassware and solvents used during the sampling procedure, as well as the sample preparation and analytical procedures. Two types of background problems exist: the first being the instrumental background, the second being the sample or volume dependant background that arises due to the sample matrix. Therefore the development and optimisation of improved analytical techniques is critical, as the application areas for trace analysis are extensive (Szczepańska *et al.,* 2018).

In some cases, the concentration of the analytes in question are simply too low for a particular analytical method, as the signal generated is too poor for accurate detection. Thus the pre-concentration of these analytes assists greatly in the performance of subsequent trace analyses (Szczepańska *et al.*, 2018). For a real-world example, and for the purposes of this study, pesticides were selected to be the focus. The maximum residue limits (MRLs) of the pesticides under study are very low, as discussed in greater detail in Section 2.2.3 for each pesticide of interest but are typically 0.05 mg/kg in South-African crops. To be able to accurately quantify an analyte at such low concentrations, extensive sample clean-up and analyte pre-concentration are typically needed.

Pesticides are used globally in large amounts on a great variety of crops. Furthermore, the pesticide target species are just as diverse and include a wide spectrum of weeds, fungi, mites and insects. South Africa has the highest consumption of pesticides in the southern hemisphere of Africa (Dabrowski *et al.,* 2014). Therefore a wide variety of pesticides are available on the market to suit the needs of both commercial and small-scale farmers, with an excess of 3000 pesticides approved for use, according to the South African Department of Agriculture, Forestry and Fisheries (DAFF) Annual Report of 2017/2018 (Buthelezi, 2018). Thus the continual development and improvement of analytical methods is needed to cater for a wide variety of sample matrices and analytes.

Furthermore, the value of South Africa's agricultural production in 2017 was estimated to be R277,6 billion (Buthelezi, 2018). Of this agricultural produce, maize is the most extensively produced crop with 2.8 million hectares of land being planted annually for cultivation (Buthelezi, 2018). It is estimated that 88% of atrazine in South Africa is used in the production of maize, while over 1 million tons per annum of this pesticide is used locally (Dabrowski, 2015). Thus the use of pesticides in South Africa is on an exceptionally large scale. Consequently, the potential health and environmental impacts on non-target species and ecosystems are vast. The pesticides that were selected as the focus of this study are atrazine, terbuthylazine, acetochlor and alachlor. All four of these pesticides are highly ranked based on their calculated weighted hazard potential, using toxicity and environmental effects in addition to usage (Dabrowski *et al.,* 2014). Of these target pesticides, atrazine has the highest weighted hazard potential in South Africa (Dabrowski *et al.,* 2014), as it is classified as an endocrine-disrupting chemical that causes health problems upon long-term exposure (Kueseng *et al.,* 2009). Furthermore, acetochlor is used as a pre-emergence herbicide that inhibits protein synthesis and from an environmental point of view, acetochlor is toxic to fish and algae (Dong *et al.,* 2009). Alachlor residues, on the other hand, can cause nasal turbinate tumours in humans (Wang *et al.,* 2015). Continuous research and development is required in South Africa, as a result of changing challenges and environmental requirements, in order to maintain healthy crop production.

The legalization of medicinal cannabis has already been introduced in several countries across the globe and as of 2017, the legalization process of cannabis for medicinal use in South Africa was initialized (Gouws, 2017), allowing applicants to apply for a cultivation licence. This brought about yet another sample matrix containing a range of pesticides that require analysis. The unique range of pesticides to be monitored on the *Cannabis sativa* and *Cannabis indica* crop is needed to ensure safe consumption by patients and recreational users alike. Consequently, in this study, a special emphasis was placed on the cannabis plant (*C. sativa and C. indica*) as a sample matrix for pesticide extractions. On 15th of May 2019, Dr Aaron Motsoaledi (the South African Minister of Health) signed a statement in the Government Gazette, No. 42477, excluding preparations that contain cannabidiol from the Related Substances Act under certain conditions. This has drastically increased the number of cannabis and cannabis related products on the South African market, thereby increasing the need for research with regards to the unique mixture of pesticides that are associated with cannabis cultivation. Because cannabis is considered to be of medicinal importance by many individuals and is subsequently used by those with a compromised immune or metabolic system, these already vulnerable individuals may be disproportionately vulnerable to the negative effects of cannabis products containing pesticides.

Due to the analytical requirements of low LODs, selectivity and efficiency as a result of the potentially high amount of pesticide usage in cannabis production, the application of self-manufactured MIP-SPEs was evaluated in this study for the extraction of the four selected pesticides from a cannabis matrix.

1.2: Aims and objectives

The aim of this study was to develop SPE cartridges containing self-manufactured MIP sorbents that can be used in the quantification of selected pesticides in a cannabis sample matrix.

The objectives of this study were to:

- Develop a suitable GC-MS method for the quantification of the selected pesticides.
- Synthesise novel MIPs, specifically:
    - Non imprinted polymer (NIP) with no template added.
    - MIPs with each individual pesticide as template.
    - MIPs synthesised with increased quantities of template, to explore the effect thereof on adsorption capacity and SPE efficiency. In this study these MIPs were referred to as enhanced adsorption capacity MIPs (EAC MIPs).
    - A multi template MIP (MTMIP) where the four pesticides were used as templates in a single MIP.
- To characterize all the synthesised polymers utilising:
    - FTIR
    - SEM
- To pack MISPE cartridges and to develop a method for the extraction of pesticides from these self-manufactured MISPE cartridges
- To determine adsorption capacities of MIPs based on these packed MISPE cartridges for:
    - Each template on their associated MIP.
    - Each template on the NIP.
    - Class specific adsorption capacities, for example, how much terbuthylazine could be adsorbed onto the atrazine MIP, thereby gaining insight into triazine pesticide class binding capacities.
- To compare the different MISPE cartridges with each other and a commercially available and commonly used C18 SPE cartridge to extract the selected pesticides from a cannabis sample matrix in terms of:
    - Selectivity
    - Efficiency
    - Cost

1.3: Justification of the study

GC-MS is one of the most powerful methods which can be used to monitor pesticide residues (Kwon *et al.,* 2012). Interest in the determination of pesticide residues has been gradually increasing, mainly due to globalization and the extent of the international food trade. The current global pesticide monitoring standards are largely determined by European countries, and involve a combination of LC-MS and GC-MS analytical methods to analyse pesticide residues at > 10 ng/g or parts per billion levels (Kwon *et al.,* 2012). Despite the chromatographic aspect of both LC and GC-MS, the fundamental problem with both analytical methods is their susceptibility to matrix effects that adversely affect the quantification of pesticide residues at low concentrations. However, many techniques and studies have been conducted to overcome and understand the matrix effects associated with analytical methods (Kwon *et al.,* 2012).

An effective way to overcome matrix effects is the use of isotopically labelled internal standards (IS). Internal standards can, however, be quite rare and expensive to acquire. Standard additions can also be used in some cases, however they require more steps for sample preparation, as each sample needs to be prepared with at least three spiking levels to obtain a calibration curve. The instrument run time, as well as the data analysis time, is increased making the method unpopular in industry mainly due to the added cost. An alternative method is to add a clean-up step such as SPE to the sample preparation, thereby reducing matrix effects in the final extract (Patnaik, 2004; Rood, 1994).

A Canadian study found that out of 144 cannabis samples taken unannounced from licenced cannabis growers, 26 tested positive for unauthorized pesticides (Moulins *et al.,* 2018). According to a literature review, it was reported that both spectral data and chromatograms of pesticide residues in cannabis extracts contain significant interferences from co-extracted cannabinoids, terpenes and other lipids (Moulins *et al.*, 2018). Traditional endcapped C18 (C18-E)-SPE has proved to be unable to remove residual lipids from the cannabis sample matrix. However enhanced matrix removal - lipid technology in combination with quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction has been shown to be an effective clean-up technique for matrices high in lipid content such as cannabis buds. The sorbents employed were C18-E & primary secondary amine (Moulins *et al.*, 2018).

There are multiple examples in the literature of methods being used for the extraction of various pesticides, including atrazine, terbuthylazine, acetochlor and alachlor mostly from soil and aqueous samples (Chen *et al.,* 2014; Dong *et al.*, 2009; Kueseng *et al.*, 2009; Wang *et al.*, 2015). Different methods of MIP polymerization have been explored and the associated effects thereof on adsorption capacities and efficiencies have been determined (Chen *et al.*, 2014). Sample matrices that have been extensively investigated with respect to pesticide residues include water and soil (Zhu *et al.,* 2005).

The use of MIPs has not been reported for the extraction of pesticides from the Cannabaceae plant family that includes cannabis (*C. sativa* and *C. indica*) and hops (*Humulus lupulus*).

As a result of the recent South African interest in cannabis and cannabis related products, the target pesticides in this study were selected due to their relevance in the South African context, as they are used in the cultivation of *C. sativa and C. indica.*

1.4: Hypothesis

The primary hypothesis is that existing methods of SPE pre-concentration of extracts with GC-MS analysis can be applied successfully to cannabis related sample matrices. Furthermore, a novel MIP based SPE method for the extraction of pesticides from cannabis can be developed, which in turn will provide selectivity and enhanced detection limits to improve these existing conventional SPE methods.

1.5: Dissertation outline

After the introduction sections in Chapter 1, the dissertation commences with a literature review in Chapter 2. The literature review outlines the use of pesticides in South Africa; why the four target pesticides used as templates in the MIP synthesis were selected; and the physicochemical properties of the target pesticides that were deemed of importance to this study. This is followed by the analysis of pesticides in terms of the usage of chromatographic techniques and mass spectrometry. The last section is a broad discussion on MIPs and of pesticide extraction methods utilising MIPs as important concepts for this particular study.

The experimental procedures employed in this study are covered in Chapter 3. MIP synthesis is discussed first. Followed by the GC-MS setup and a method for the quantification of the selected pesticides and the effect of silanization of the GC inlet liner on analyte peak shape. MIP adsorption capacity determination and characterization methodologies are described next. This is followed by MIP size fractionation and template removal procedures in preparation for MISPE packing. Lastly, the SPE method and the associated optimization procedures related to separation and elution efficiency are discussed. The results and discussion are covered in Chapter 4. In this chapter, selected chromatograms, mass spectra, SEM images and FTIR spectra are presented and discussed.

In the conclusion and future work presented in Chapter 5, a final comparison is drawn between the synthesised polymer SPEs and the commercial C18 SPE. The comparison is based on environmental

effects, cost, selectivity and adsorption capacity. Several recommendations that are based on the observations and possible extensions of this study, are also included for future research.

The final section of the dissertation (the Appendix) contains Certificates of Analysis (COAs) for standards and licences to possess cannabis for analytical testing purposes obtained from the South African Health Products Regulatory Authority (SAHPRA). Fragmentation patterns of some pesticide analytes are also presented.

Chapter 2 : Literature review

In this chapter, a general background to pesticide classes and the use thereof in South Africa is reviewed. Several extraction methods commonly used for pesticide extraction from plant material are discussed followed by chromatographic and mass spectrometric techniques used for pesticide analysis. MIPs are described, both in terms of their synthesis, characterization, and use for extraction of the target pesticides. As it is of importance to the justification of the study, the use of cannabis and its legal status in South Africa are also discussed.

2.1: Introduction

The earliest procedures for residual pesticide analysis were based on extraction thereof from foodstuff by liquid-liquid extraction. According to literature, the first publication dealing with the extraction and clean-up of multiresidue pesticides, including organochlorine, organophosphate, organonitrogen, and hydrocarbon pesticides was published in 1975 by Luke *et al.* (as reported in Rahman *et al.,* 2017; Dušek *et al.,* 2018). Acetone, acetonitrile or ethyl acetate extraction solvents were first utilised, followed by liquid–liquid extraction with dichloromethane and then further clean-up was required using adsorbent column chromatography, for example a silica or alumina column. Later the "Dutch mini-Luke" method was developed to avoid the use of chlorinated solvents (Dušek *et al.,* 2018). GC-MS analysis typically followed such a sample clean-up. To extend the scope of analytes to include more polar pesticides and to reduce the number of sample preparation steps required, solid phase extraction (SPE) subsequently replaced the silica column clean-up step, using a mixture of primary and secondary amines (PSAs) with graphitized carbon black (Rahman *et al.*, 2017). SPE and several other extraction methods are discussed in detail in Section 2.4. A more modern approach involves the use of quick, easy, cheap, effective, rugged, and safe (QuEChERS) extraction salts for sample preparation followed by LC-MS/MS analysis. These techniques are widely used for the extraction and analysis of multiresidue pesticides, including the four selected for this study (Mastovska *et al.,* 2017; Dušek *et al.,* 2018) and the cannabis sample matrix (Atapattu and Johnson, 2020; Moulins *et al.,* 2018; Taylor & Birkett, 2020).

2.2: Pesticides

The EPA defines pesticides in the following three manners: 1) Any substance or mixture used for preventing, destroying, repelling or mitigating any pest. 2) Any substance or substances intended for use as a plant regulator, defoliant, or desiccant. 3) Any substance used as a nitrogen stabilizer (Leahy *et al.,* 2014).

Pesticides are commonly utilized in agriculture for crop protection and to protect human and animal health from pests. Pesticides are classified as insecticides, herbicides, rodenticides, fungicides, nematicides, molluscicides and acaricides based on their field of use. Commercial formulations are often mixtures of pesticide classes and active ingredients (Anthony *et al* ., 2011).

2.2.1: Use of pesticides in South Africa

South Africa is the largest consumer of pesticides in sub-Saharan Africa (Dabrowski *et al.*, 2014). Atrazine, terbuthylazine, acetochlor and alachlor are all in the top 25 pesticides ranked by weighted hazard potential (WHP), which is calculated based on the amount of pesticide used annually in South Africa, the toxicity potential and environmental hazard potential thereof (Dabrowski *et al.,* 2014). The maximum residue limits (MRLs) and on which crops in South Africa the pesticides are applied and subsequently are monitored are listed in Table 2.1. The MRL of a particular pesticide may differ for different crops as different amounts of produce may typically be consumed for each crop. The South African limits are published in the Government Gazette, GNR.246 of 11 February 1994 (Department of National Health and Population Development, 2012). No specific regulation has to date been published for cannabis in South Africa in this regard.

Table 2.1: Top 25 most used pesticides in South Africa along with the MRLs and typical analytical methods employed for each pesticide (Alder *et al.,* 2006; Cullum & Schuhn*,* 2013; Dabrowski *et al.,* 2014, GNR.246 of 11 February 1994)

	Active ingredient	Class	Chemical class	Typical analytical method	Common crops to which it is applied and related maximum South African residue limits in mg/kg (ppm)
1	Glyphosate	Herbicide	Organophosphate	HPLC-MS (Cullum & Schuhn, 2013) LC-MS/MS (Alder *et al.,* 2006) GC-MS (Börjesson & Torstensson, 2000)	Sugarcane (0.5)
2	Mancozeb	Fungicide	Metal- organic compound	HPLC-MS (Chi-Chu *et al.,* 1996)	Apples, apricots, bananas, beans, boysenberries, citrus, grapes, guavas, mangoes, olives, papayas, peaches, pears, peppers, plums and tomatoes (3.0)

	Active ingredient	Class	Chemical class	Typical analytical method	Common crops to which it is applied and related maximum South African residue limits in mg/kg (ppm)
3	Sulphur	Fungicide	Inorganic	GC-ICP-MS (Geiger *et al.,* 2007)	Apples, apricots, avocados, bananas, beans, boysenberries, citrus, cucurbits, grapes, mangoes, papayas, peaches, pears, peas, peppers, plums and tomatoes (50.0)
4	Copper oxychloride	Fungicide	Inorganic	ICP-MS (Schutte *et al.,* 2012)	Apples, apricots, avocados, beans, boysenberries, celery, cherries, citrus, coffee, granadillas, grapes, guavas, lettuce, mangoes, olives, peaches, pears, peppers, plums, strawberries and tomatoes (20.0)
5	Atrazine	Herbicide	Triazine/ Organochloride	GC-MS (Carter, 1996) (Rocha, 2008)	Maize, sorghum and sugarcane (0.05)
6	Terbuthylazine	Herbicide	Triazine / Organochloride	GC-MS (Carter, 1996)	Maize, peas and sorghum (0.05)
7	Acetochlor	Herbicide	Chloroacetanilides/ Organochloride	GC-MS (Rocha, 2008)	Groundnuts and sugarcane (0.02) Cotton seed, maize and sorghum (0.05)
8	Metolachlor	Herbicide	Chloroacetanilides	GC-MS (Rocha, 2008)	Cotton seed, dry beans, green beans, groundnuts, kidney beans, maize, potatoes, sorghum, soya beans, sugarcane and sunflower seed (0.05)
9	2,4-D-amine (2,4-Dichlorophenoxyacetic acid)	Herbicide Plant Growth regulator (PGR)	Organochloride	LC-MS/MS (Alder *et al*. 2006) GC-MS (EPA 8151A)	Barley, maize, rye, sorghum, sugarcane and wheat (0.5) Citrus (2.0) Potatoes (0.1)
10	Paraquat	Herbicide	Bipyridine	HPLC/UV (Restek Corporation app. note 580006)	Cotton seed (0.2) Maize (0.05) Sugarcane (0.5)

	Active ingredient	Class	Chemical class	Typical analytical method	Common crops to which it is applied and related maximum South African residue limits in mg/kg (ppm)
11	Alachlor	Herbicide	Chloroacetanilide	GC-MS (Rocha, 2008)	Broccoli, Brussels sprouts, cabbage, maize, potatoes, soya beans and sunflower seed (0.1) Groundnuts, pineapples and sugarcane (0.05)
12	MCPA (2-methyl-4-chlorophenoxyacetic acid)	Herbicide	Organochloride	GC-MS (EPA 8151A)	Barley, maize, potatoes, rye, sorghum, sugarcane and wheat (0.1)
13	Ethylene-dibromide	Fumigant	Organobromine	GC-MS (Haib, 2003)	N/A (Not used on crops)
14	Imidacloprid	Insecticides	Organochloride	LC-MS/MS (Alder *et al.,* 2006)	Apples (0.2) Citrus (0.5) Cucurbits, grapes, maize and cotton seed (0.05) Sorghum, sunflower seed and wheat (0.02) Tomatoes (0.1)
15	MSMA (Monosodium methyl arsenate)	Herbicide	Arsenic	ICP-MS (Schutte *et al.*, 2012)	Sugarcane (0.5)
16	Potassium-phosphate	Fungicide	Inorganic Phosphate	ICP-MS (Rui *et al.,* 2012)	Barley, maize, potatoes, rye, sorghum, sugarcane and wheat (0.1)
17	Cyanamide	Fungicide & Insecticide	Amine	ICP-MS (Schutte *et al.*, 2012)	Apples, grapes and kiwifruit (0.05)
18	EPTC (s-ethyl dipropylthiocarbamate)	Herbicide	Carbamate Pesticides	GC-MS (Goodman, 2007)	Dry beans, green beans, kidney beans, maize, potatoes, sugarcane, sunflower seed, sweet corn and sweet potatoes (0.05)
19	Copper hydroxide	Fungicide & Insecticide	Inorganic	ICP-MS (Schutte *et al.*, 2012)	Barley, maize, potatoes, rye, sorghum, sugarcane and wheat (1.0)
20	Trifluralin	Herbicide	Organic Fluoride	LC-MS/MS (Fang *et al.*, 2006)	Cabbage, chillies, cowpeas, Dry beans, groundnuts, kidney beans, soya beans,

	Active ingredient	Class	Chemical class	Typical analytical method	Common crops to which it is applied and related maximum South African residue limits in mg/kg (ppm)
					sunflower seed and tomatoes (0.05) Carrots (1.0)
21	Copper-carbonate	Algaecide, herbicide, wood preservative	Inorganic	ICP-MS (Schutte *et al.*, 2012)	N/A- not used on edible crops
22	Chlorpyrifos	Insecticide	Organophosphate	LC-MS/MS (Fang *et al.*, 2006)	Apples, apricots, carrots, lettuce, mealies (green), peaches, pears, plums, potatoes and wheat (0.05) Bananas (1.0) Grapes and tomatoes (0.5) Citrus (0.3)
23	Chlorothalonil	Fungicide	Organochloride	GC-MS and LC-MS (Chaves, 2008)	Beans and tomatoes (3.0) Peas (0.3) Groundnuts and potatoes (0.1)
24	Terbufos	Insecticide & nematicide	Organophosphate	GC-MS (US EPA 8140)	Citrus, groundnuts, mealies (green), potatoes, sorghum and sunflower seed (0.1) Dry beans (0.05)
25	s-Metolachlor	Herbicide	Organochloride	LC-MS/MS (Fang *et al.*, 2006)	Cotton seed, dry beans, green beans, groundnuts, kidney beans, maize, potatoes, sorghum, soya beans, sugarcane and sunflower seed (0.05)

2.2.2: Classes of pesticides

As already mentioned, pesticides can be divided into classes based on their applications or chemical functional groups, depending on the context. There are seven common application classes that include the following: insecticides, herbicides, fungicides, rodenticides, acaricides, molluscicides and nematicides (Anthony *et al.,* 2011). Each of these application classes contain several chemical groups, for example, insecticides can be subdivided into organophosphate, carbamates, organochlorines,

pyrethroids and substituted ureas. Herbicides are classified into chlorinated phenoxy acids, substituted ureas, triazines, uracils, quaternary ammonium compounds, carbamates, carboxylic acids and esters. Dithiocarbonate complexes with manganese, nickel and zinc are commonly used as fungicides. Organic and inorganic compounds of copper and mercury are also used as fungicides. There are three notable chemical classes for rodenticides, namely, phosphines, thallium salts and coumarin anticoagulants. Warfarin and dicumarol are potent anticoagulants that inhibit the cofactor function of vitamin K (Moffat *et al.*, 2011).

There are some pesticides that cannot be categorized into a single class, as they contain more than one functional group or categoric feature (Alder *et al.,* 2006). Figure 2.1 displays the classes of pesticides as they are commonly divided into based on chemical properties. As GC-MS was to be used in this study, colour coding has been used in Figure 2.1 to show the compatibility of GC-MS analytical methods for each pesticide compound class, where green indicates that GC-MS is suitable for many pesticides of that class, and there are many publications available in this regard. Yellow is used where there are examples of GC-MS analysis in the literature, but the methodology is clearly not ideal. Red indicates that there are minimal examples of pesticides in this class that can be analysed by GC-MS and few publications where GC-MS analysis was employed for their analysis. Biopesticides were not included in Figure 2.1, because of the large variety of chemicals this class contains and they therefore cannot fall into a single chemical class. Biopesticides include compounds extracted from plants and may even refer to the genetic modification of plants making them more resistant to pests, as discussed in greater detail in Section 2.2.2.e.

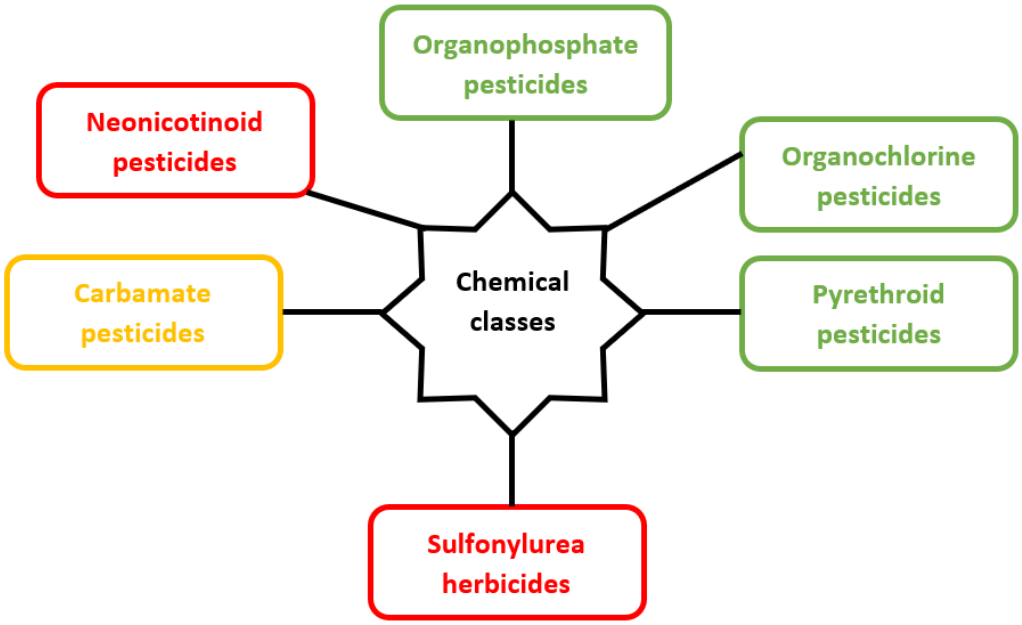


Figure 2.1: Pesticide classes based on functional groups in the chemical structure thereof. Colour coding refers to GC-MS analysis compatibility where green means compatible, yellow is partially compatible and red is incompatible

Figure 2.2 indicates pesticide classes as per their application or target pests. Biopesticides are included, but they are not application specific and include a wide variety of compounds that can be applied to control a variety of pests.

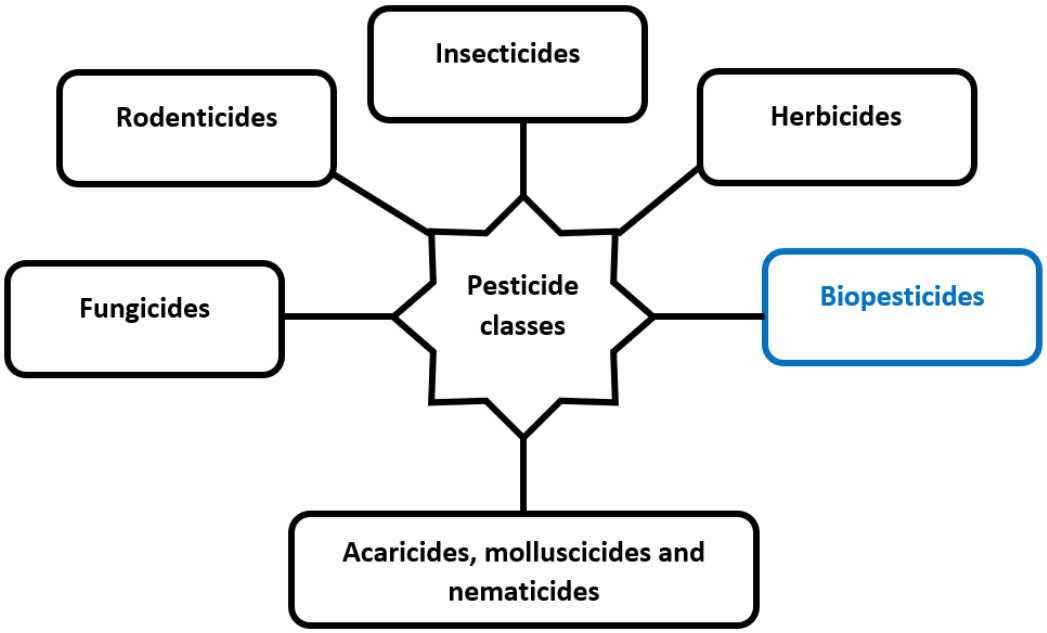


Figure 2.2: Pesticide classes as defined by their application. Biopesticides are in a different colour as they can be applied to control multiple organisms

2.2.2.1: Organophosphate pesticides

Glyphosate is the world's biggest selling and most widely used herbicide, obviously making the organophosphate class a priority for analysis (Cullum & Schuhn, 2013). Glyphosate has a relatively low toxicity and health risk when compared to other pesticides, this also gives rise to its extensive use (Dabrowski *et al.,* 2014). Long term exposure can, however, lead to endocrine effects in mammals (Cullum & Schuhn, 2013). As a cholinesterase inhibitor, most organophosphates are toxic to mammals and humans (Zhu *et al*., 2005). Despite these associated potential negative effects, organophosphate insecticides are still one of the most widely used pesticide classes (Oberemok *et al.,* 2015) and are applied to control a broad range of pests on a range of produce including cotton, rice, tobacco, sorghum, sugarcane and selected vegetables (Zhu *et al*., 2005). Limits of quantitation displayed in Table 2.2 relate to various target matrices which have been achieved by GC-MS analysis.

Table 2.2: Commonly used and analysed organophosphate pesticides that can be analysed by GC-MS with examples of limits of quantitation that have been achieved

Organophosphate	Class	Limit of quantitation (LOQ)	Matrix
Acephate	Insecticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Azinphos-Methyl	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water

Organophosphate	Class	Limit of quantitation (LOQ)	Matrix
Carbofuran	Insecticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Chlorfenvinphos	Insecticide and acaricide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Chlorpyrifos	Insecticide, acaricide and miticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Coumaphos	Insecticide and miticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Crotoxyphos	Insecticide	1000 ng/mL (Alder *et al.,* 2006)	Natural water
Demeton	Insecticide	1 ng/ul (Wheeler, 2011)	Spiked standard
Diazinon	Insecticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Dichlorvos	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Dicrotophos	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Dimethoate	Insecticide and acaricide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Dioxathion	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Disulfoton	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
EPN	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Ethion	Insecticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Fenamiphos	Insecticide	1000 ng/mL (Alder *et al.,* 2006)	Natural water
Fenitrothion	Insecticide	100 ng/mL (Alder *et al.,* 2006) 5.0 ug/kg (Kolberg *et al.,* 2011)	Natural water Dry flour
Fonofos	Insecticide	1 ng/mL (Alder *et al.,* 2006)	Natural water
Malathion	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Methamidophos	Insecticide	10 ng/g (Zou & Zhai, 2015)	Tomato
Methidathion	Insecticide	10 ng/g (Zou & Zhai, 2015)	Tomato
Methyl parathion	Insecticide and acaricide	10 ng/g (Zou & Zhai, 2015)	Tomato
Mevinphos	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Monocrotophos	Insecticide	1000 ng/mL (Alder *et al.,* 2006)	Natural water
Naled	Insecticide	10 000 ng/mL (Alder *et al.,* 2006)	Natural water
Parathion	Insecticide and acaricide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Phorate	Insecticide and acaricide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Phosalone	Insecticide and acaricide	1000 ng/mL (Alder *et al.,* 2006)	Natural water
Phosmet	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Phosphamidon	Insecticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
TEPP (Tetraethyl pyrophosphate)	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Terbufos	Insecticides and nematicides	10 ng/mL (Alder *et al.,* 2006)	Natural water
Tetrachlorvinphos	Insecticide (Fleas and Ticks)	10 ng/mL (Alder *et al.,* 2006)	Natural water

2.2.2.2: Organochlorine insecticides

Atrazine was ranked as the pesticide with the highest weighted hazard potential (WHP) in South Africa (Dabrowski *et al.,* 2014; Dabrowski, 2015). Several chromatographic methods have been reported in the literature for the analysis of chloroacetamide herbicides, including atrazine, acetochlor and

alachlor (Anthony *et al.,* 2011). The methods include GC-MS and LC-MS (Wang *et al.*, 2015), however only GC-MS based methods are included in Table 2.3.

Table 2.3: Commonly used and analysed organochlorine pesticides that can be analysed by GC-MS with examples of limits of quantitation that have been achieved for various sample matrices

Organochlorine	Class	Limit of quantitation by GC-MS	Matrix
Aldrin	Insecticide	1 ng/mL (Alder *et al.,* 2006) 5 ug/kg (Kolberg *et al.,* 2011)	Natural water Dry flour
alpha-BHC	By-products of Lindane	-	-
beta-BHC		-	-
delta-BHC		-	-
gamma-BHC (Lindane) (Hexachlorocyclohexane)	Insecticide	5 ug/kg (Kolberg *et al.,* 2011)	Dry flour
***cis*-Chlordane**	Termite control	1 ng/mL (Alder *et al.,* 2006)	Natural water
***trans*-Chlordane**			Natural water
Chlorpyrifos-ethyl	Insecticide, Acaracide and Miticide	5 ug/kg (Kolberg *et al.,* 2011)	Dry flour
Chlorpyrifos-methyl	Insecticide	10 ug/kg (Kolberg *et al.,* 2011)	Dry flour
4,4'-DDD	Insecticide	1 ng/mL (Alder *et al.,* 2006)	Natural water
2,4'-DDE	Insecticide	1 ng/mL (Alder *et al.,* 2006)	Natural water
4,4'-DDE	Insecticide	1 ng/mL (Alder *et al.,* 2006)	Natural water
4,4'-DDT	Insecticide	1 ng/mL (Alder *et al.,* 2006)	Natural water
Dieldrin	Insecticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Endosulfan Alpha	Insecticide	100 ng/mL (Alder *et al.,* 2006) 5.0 ug/kg (Kolberg *et al.,* 2011)	Natural water Dry flour
Endosulfan Betha	Insecticide	100 ng/mL (Alder *et al.,* 2006) 5.0 ug/kg (Kolberg *et al.,* 2011)	Natural water Dry flour
Endosulfan sulfate	Insecticide	100 ng/mL (Alder *et al.,* 2006) 5.0 ug/kg (Kolberg *et al.,* 2011)	Dry flour Dry flour
Endrin	Insecticide, Rodenticide and Piscicide	10 ng/mL (Alder *et al.,* 2006) 5.0 ug/kg (Kolberg *et al.,* 2011)	Natural water Dry flour
Heptachlor	Insecticide	1 ng/mL (Alder *et al.,* 2006)	Natural water
Heptachlor epoxide (Isomer B)	Insecticide	10 ng/mL (Alder *et al.,* 2006)	Natural water
Hexachlorobenzene	Fungicide	1 ng/mL (Alder *et al.,* 2006)	Natural water
Isodrin (Isomer of Aldrin)	Insecticide	5 ug/kg (Kolberg *et al.,* 2011)	Dry flour
Methoxychlor (Dimethoxy- DDT)	Insecticide	100 ng/mL (Alder *et al.,* 2006)	Natural water
Mirex	Insecticide	1 ng/mL (Alder *et al.,* 2006)	Natural water
Tetradifon	Insecticide	10 ng/mL (Alder *et al.,* 2006) 5.0 ug/kg (Kolberg *et al.,* 2011)	Natural water Dry flour

Commercial SPE cartridges are available to pre-concentrate solvent extracts containing organochlorine pesticides from plant material such as the C18 Silica-Based cartridges. The C18 SPE cartridges are commonly used for concentrating pesticides, herbicides, hydrocarbons, and other

organic contaminants in water (Lingam *et al.,* 2012). It is stated in literature that the C18 sorbent is extremely effective at retaining non-polar compounds and is in fact the most hydrophobic silica bonded sorbent available. Additionally it is effective for desalting aqueous mixtures (Chen *et al.*, 2014; Lingam *et al.*, 2012).

2.2.2.3: Pyrethroid pesticides

Allethrin was synthesised in 1949, leading to the subsequent synthesis of many pyrethroid pesticides based on pyrethrin, the base chemical structure (Oberemok *et al.*, 2015). Pyrethroids have been used more often in recent years as a replacement for organophosphate pesticides because of concerns regarding water quality (Hldik *et al.,* 2009). As insecticides, pyrethroids are very toxic to sediment dwelling organisms and are used in agriculture and urban environments alike. Pyrethroids have low toxicity to warm blooded mammals, making them popular for use because the human health risk associated with these insecticides is consequently reduced. Pyrethroids are hydrophobic and adsorb to particulate matter present in natural waters (Hldik *et al.,* 2009).

SPE based sample preparation has been used in the analysis of pyrethroid pesticides. The sorbents include C8, C18 and HLB (Hydrophilic-Lipophilic-Balanced)(Hldik *et al.,* 2009). Figure 2.3 illustrates structures of pyrethroid pesticides, extracted from water and sediment samples, that were analysed using GC-MS (Hldik *et al.,* 2009).

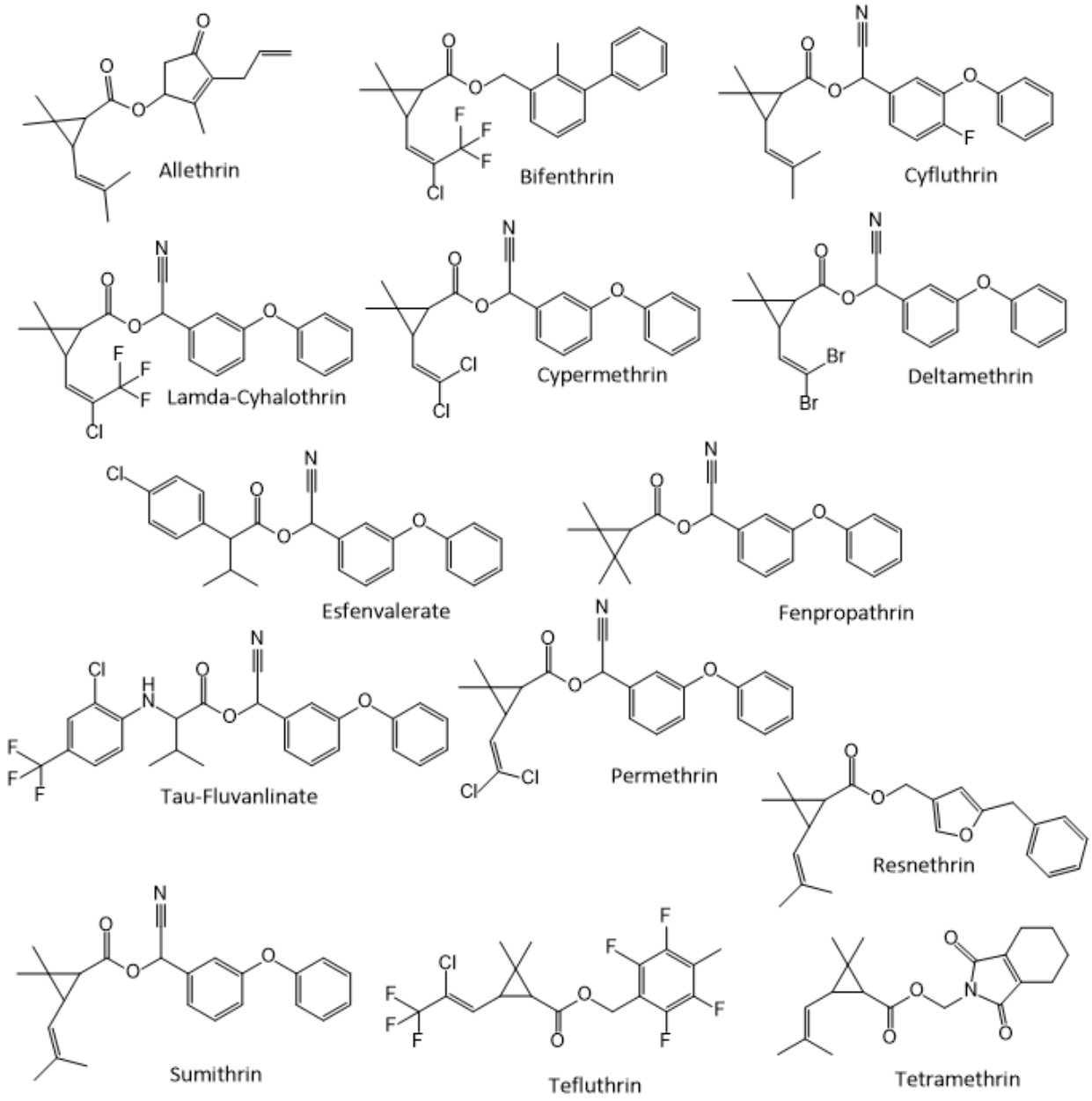


Figure 2.3: Structures of 14 examples of pyrethroid pesticides that have been analysed utilising GC-MS (Hldik, 2009)

2.2.2.4: Sulfonylurea herbicides

Sulfonylureas inhibit the plant enzyme, acetolactate synthase, resulting in impaired branch chain amino acid synthesis (Headley *et al.,* 2010). Sulfonylurea herbicides are popular as they provide good selectivity and can kill specific plant types while leaving the rest of the crop intact. Very low application rates or concentrations are required leading to favourable environmental and toxicological properties. Physicochemical properties of sulfonylurea herbicides include low vapor pressure, high water

solubility (200 g/L for mesopleuron-methyl), and high phytotoxicity (Headley *et al.*, 2010). Their water solubility and phytotoxicity may cause significant environmental effects if large quantities of these pesticides are introduced into the environment. Generally, environmental concentrations of sulfonylureas herbicides are a challenge to quantify due to the low application concentration (5–40 g/ha) (MacBean, 2012). LC-MS/MS is the most popular quantitation method for sulfonylurea herbicides with detection limits typically ranging from 1.3 to 7.2 pg on-column (Headley *et al.*, 2010). Figure 2.4 shows examples of chemical structures of common sulfonylurea herbicides.

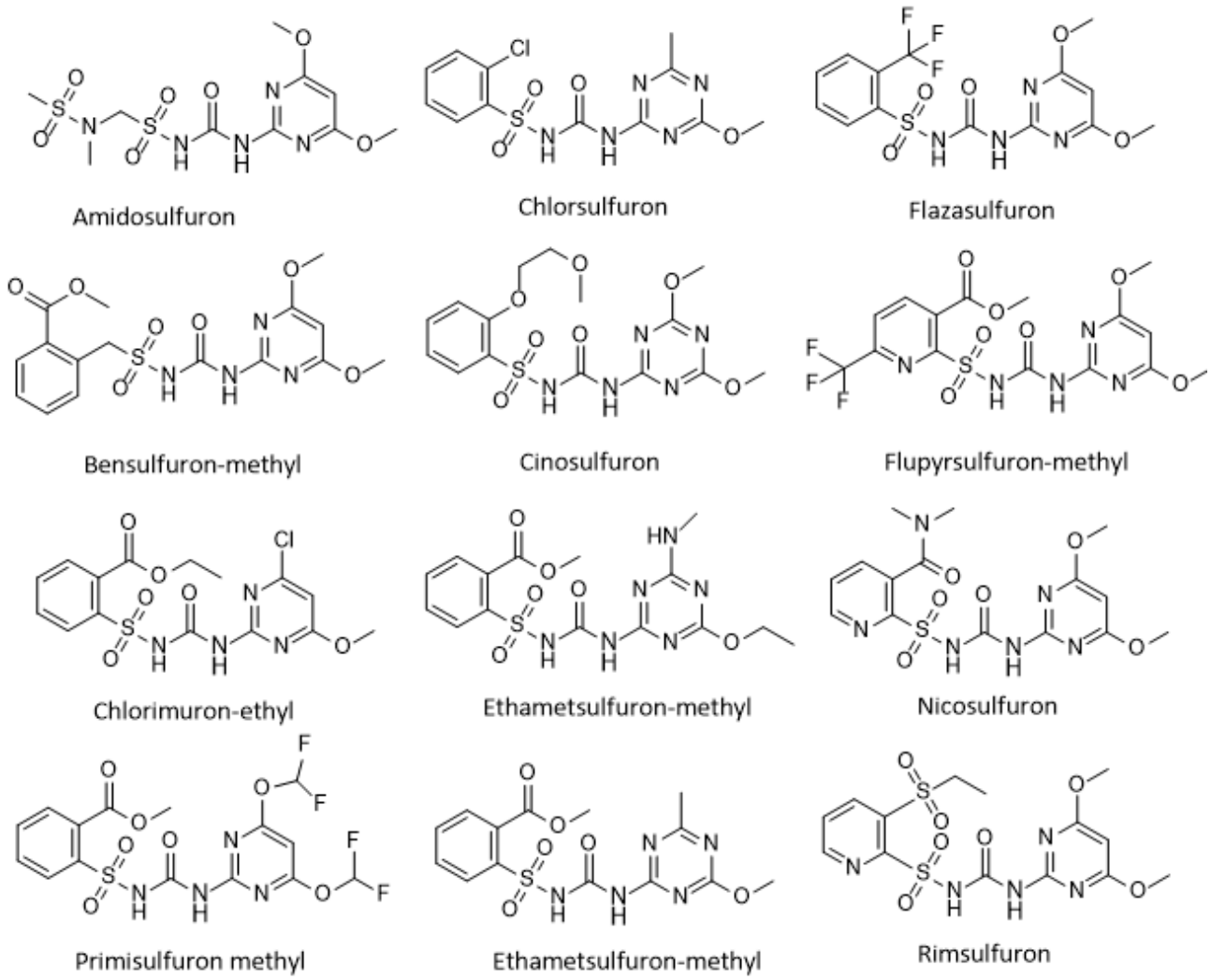


Figure 2.4: Chemical structures of twelve examples of sulfonylurea herbicides (Headley et al., 2010)

Methods for the extraction of sulfonylurea herbicides from water samples typically utilise the partitioning thereof between the aqueous phase and a water-immiscible solvent (via LLE), although SPE can also be used. According to a review article there are literature examples of MIPs being used to extract sulfonylurea herbicides from soil samples (Whitcombe *et al.*, 2014). Additionally, C18, N-vinyl-pyrrolidone polymer, and polystyrene–divinylbenzene (PS–DVB) materials have been used as

sorbents for SPE of some sulfonylurea herbicides from pond and river water samples (Alder *et al.,* 2006). LC-MS/MS is the most popular chromatographic technique for the quantification of sulfonylureas for which the limits of quantification (LOQ) are approximately 0.1 mg/L from natural river water (Headley *et al.*, 2010).

2.2.2.5: Biopesticides

The United States EPA states that biopesticides "include naturally occurring substances that control pests (biochemical pesticides), microorganisms that control pests (microbial pesticides), and pesticidal substances produced by plants containing added genetic material plant-incorporated protectants (PIPs) (Leahy *et al.*, 2014). The oldest used pesticides were botanical preparations from *Dalmatian pyrethrum* flowers, that contain 1.5% of pyrethrin, which were applied in ancient China and during the Middle Ages in Persia (Oberemok *et al.*, 2015).

Biopesticides fall into three major categories: microbial pesticides, plant-incorporated protectants and biochemical pesticides. Microbial pesticides utilize a microorganism such as a bacterium, fungus, virus, protozoan or alga as the active ingredient. Microbial pesticides can control a variety of pests, although each microbe is relatively specific for its target. The most widely known microbial pesticides are varieties of the bacterium *Bacillus thuringiensis*, which can control insects in cabbage and potato by producing a protein that is harmful to the specific insect pest (Sharma & Malik, 2012). PIPs are pesticidal substances that plants produce from genetic material that has been added to the plant through gene editing technology. Biochemical pesticides are naturally occurring substances such as plant extracts, fatty acids or pheromones that can kill pests. Biochemical pesticides can interfere with growth or mating, such as plant growth regulators (Sharma & Malik, 2012).

Examples of biopesticides extracted from plants include limonene and linalool which are naturally occurring terpenes that can be used to control fleas, aphids, mites, fire ants, several types of flies, paper wasps and house crickets. Rotenone is an odourless, colourless, crystalline isoflavone used as a broad-spectrum insecticide, which can be extracted from several plant species in which it naturally occurs. Pyrethrum refers to a daisy, from which pyrethrins can be extracted as previously mentioned. Pyrethrins are commonly used to control mosquitoes, fleas, flies, moths and ants (Sharma & Malik, 2012).

2.2.2.6: Carbamate pesticides

More than 50 compounds are classified as carbamate pesticides, which are used in agriculture as insecticides, fungicides, herbicides, nematicides and sprout inhibitors, and in household products for the control of household pests. These pesticides are derived from carbamic acid. The mode of activity is variable in terms of target sites thus the carbamate compounds are not very specific resulting in

mammalian toxicity and environmental persistence (Fishel, 2015). Carbamate pesticides were introduced in 1956, with carbaryl being the first carbamate pesticide. Carbaryl has low mammalian toxicity, both dermally and orally, and the wide control spectrum thereof has resulted in its extensive private use on lawns and gardens (Fishel, 2015; Struger *et al.,* 2016). Figure 2.5 and 2.6 show the structure of carbamic acid and carbaryl.

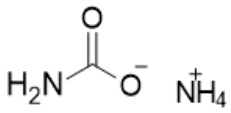


Figure 2.5: Chemical structure of carbamic acid monoammonium salt (NIST, 2014)

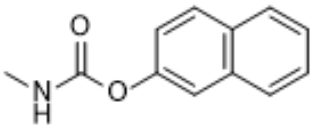


Figure 2.6: Chemical structure of carbaryl pesticide, the first carbamate pesticide introduced in 1956 (NIST, 2014)

Another example of carbamate is metalaxyl (Figure 2:7), a fungicide of the benzenoid chemical class. It controls diseases caused by air- and soil-borne *peronosporales.*

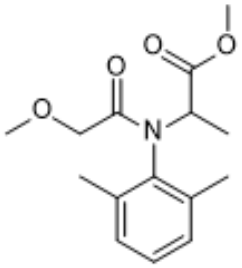


Figure 2.7: Chemical structure of metalaxyl chemical structure (NIST, 2014)

Carbamate pesticides kill insects based on their ability to inhibit acetylcholinesterase, and other esterase enzymes in the nervous system affecting nerve impulse transmission (Struger *et al.*, 2016). Carbamates are hydrolysed in the liver and the degradation products are then extracted by the kidneys. Pulmonary edema and respiratory depression are common symptoms caused by carbamate exposure (Fishel, 2015). Some carbamates can be translocated within plants, resulting in an effective systemic treatment (Fishel, 2015; Struger *et al.*, 2016). Regarding human toxicity, the main routes of carbamate exposure are through inhalation, ingestion or dermal permeation, of which the dermal route is the least toxic. Carbofuran (Figure 2.8) has a rat oral lethal dose 50 % (LD50) of 8 mg/kg, compared to a rat dermal LD50 of >3000 mg/kg (Fishel, 2015).

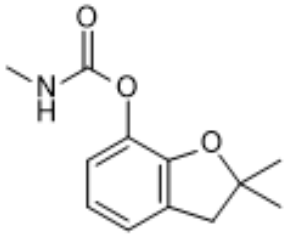


Figure 2.8: Chemical structure of carbofuran chemical structure (NIST, 2014)

The vapour pressure of carbamates is generally very low (Struger *et al.*, 2016), thus making them incompatible for GC-MS analysis. Carbamates are highly water soluble, and aqueous systems are an effective mode of environmental transport. They rapidly decompose by photodegradation and/or photodecomposition because of their light absorption properties in aqueous solutions (Struger *et al.*, 2016). From an environmental point of view, most carbamate pesticides are toxic to *Hymenoptera* (this order of insects includes the honey bee), thus precautions should be taken to ensure foraging honey bees are not affected (Fishel, 2015).

2.2.2.7: Neonicotinoid pesticides

The first neonicotinoid pesticide was introduced in the 1990s, called imidacloprid (Figure 2:9). Neonicotinoids can be divided into three chemical classes namely N-nitroguanidine, nitromethylene and N-cyanoamidines, where imidacloprid falls in the N-nitroguanidine class. Imidacloprid is a nicotinic acetylcholine receptor agonist; it binds to the receptor, causing nervous stimulation at low concentrations, but receptor blockage, paralysis and death at higher concentrations (Goulson, 2013). The nicotinic acetylcholine receptor is made up of a group of polypeptides that respond to the neurotransmitter, for comparison of the chemical structures refer to acetylcholine (Figure 2.10) and imidacloprid (Figure 2.9) that both stimulate this receptor. Neonicotinoids were the most widely used insecticides in 2013 worldwide and make up approximately one quarter of all insecticides used (Goulson, 2013; Oberemok *et al.*, 2015). They can travel through plant tissue and can thus ensure protection of the entire plant system from root to leaves. Neonicotinoids are mostly used as seed dressings (Goulson, 2013).

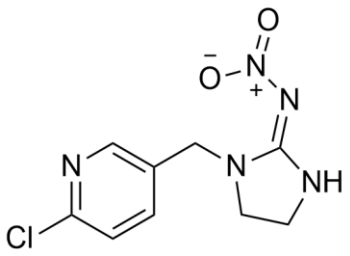


Figure 2.9: Chemical structure of imidacloprid, the first neonicotinoid introduced commercially (NIST, 2014)

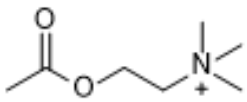


Figure 2.10: Chemical structure of acetylcholine, the natural stimulator for the nicotinic acetylcholine receptor, being targeted by imidacloprid (NIST, 2014)

Neonicotinoids act as neurotoxins and are effective against arthropods (invertebrate animals having an exoskeleton). Neonicotinoids are selectively more toxic to insects than to vertebrates, which is a major factor in their popularity as insecticide (Oberemok *et al.*, 2015). The LD50 for imidacloprid and clothianidin (Figure 2.11) in honeybees can be as little as 4 and 5 ng per insect respectively, this in comparison to DDT is about 1/10 000 of the LD50 for the same insects (Goulson, 2013). Neonicotinoids are easy to apply, as they are readily soluble in water and are easily absorbed by plants, either by their roots or leaves (Goulson, 2013).

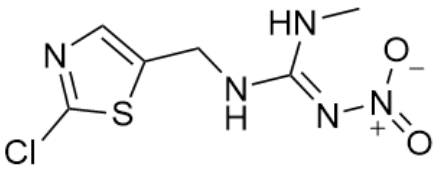


Figure 2.11: Chemical structure of c*lothianidin* (NIST, 2014)

From an environmental point of view, given the scale of application, their persistence in soils and easy water solubility, almost all organisms inhabiting arable environments will be exposed to neonicotinoids. According to a literature review on neonicotinoid insecticides, there have been numerous studies on the toxicity of neonicotinoids to a wide range of organisms, including mammals, birds, fish, insects, crustacean, molluscs and annelids. Some insects have evolved resistance to neonicotinoids because of their preventative and wide spread use (Goulson, 2013).

2.2.3: Pesticides of interest to this study

2.2.3.1: Triazine pesticides

Triazines belong to the 1,3,5 triazine group, and thus contain several nitrogen groups giving a satisfactory flame ionization detector (FID) sensitivity. Triazines are also readily amenable to GC-MS analysis and have characteristic mass spectra from electron ionization (EI) fragmentation. LC-MS is also frequently used to study triazines, particularly their degradation products, namely hydroxy- and des-alkyl triazines (Anthony *et al.,* 2011).

Atrazine

Atrazine is classified as an organochlorine insecticide, part of the triazine chemical group, and is a synthetic pesticide widely used all over the world. It belongs to a group of chlorinated hydrocarbon derivatives, which have vast number of applications in the chemical industry and in agriculture. Atrazine is used to control grassy and broadleaf weeds in sugarcane, wheat, conifers, sorghum, nuts and corn (Singh *et al.,* 2018). Figure 2.12 shows the chemical structure of atrazine containing a chlorine functional group. Atrazine is known for high toxicity, slow degradation and bioaccumulation (Jayaraj *et al.,* 2016). Ingestion of 100 g of atrazine can lead to a coma, circulatory collapse, metabolic acidosis and gastric bleeding. This may be followed by renal failure, hepatic necrosis and a disseminated intravascular coagulopathy that may prove fatal (Anthony *et al.,* 2011).

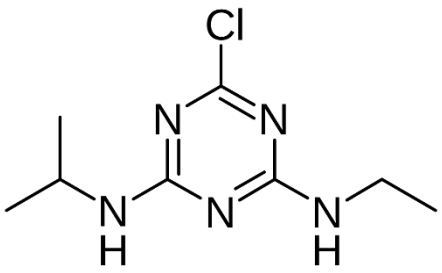


Figure 2.12: Chemical structure of atrazine (NIST, 2014)

Atrazine is the highest ranked pesticide in terms of weighted hazard potential in South Africa (Dabrowski *et al.*, 2014). It is not the most toxic pesticide used in South Africa, but it is rather due to the large quantities used that gives it the highest weighted hazard potential (Dabrowski *et al.,* 2014). The maximum residue limit (MRL) for atrazine is 0.05 mg/kg for South African crops (Department of National Health and Population Development, 2012). Atrazine acts on the growth, enzymatic processes and photosynthesis of the targeted weeds. Atrazine primarily enters the environment through its use in spraying of crop farms to control grassy and broadleaf weeds. It is sprayed both before and after the planting of crops (Dabrowski, 2015).

Unfortunately, atrazine exerts side effects in non-target aquatic fauna and flora such as mutagenicity, genotoxicity, defective cell division, erroneous lipid synthesis and hormonal imbalance (Hayes *et al.,* 2010). It has threatened the sustainability of agricultural soils due to detrimental effects on resident soil microbial communities. In postmenopausal women, endocrine disrupting effects have been observed if atrazine exposure occurs (Singh *et al.*, 2018). In other studies, when primary human hepatocytes and human neuroblastoma cells were exposed to atrazine, the observed effects included apoptosis or cell death and aromatase (also called estrogen synthetase or estrogen synthase) hormonal expression (Singh *et al.*, 2018). Atrazine is classified as an endocrine-disrupting chemical that causes health problems at high dosages or after long-term exposure. Studies have shown atrazine to cause blood hormone changes, affecting ovulation in animals. Heart, liver and kidney damage was also observed in animals exposed to atrazine (Singh *et al.,* 2018). Additionally atrazine has been shown to induce sex reversal in male African clawed frogs (*Xenopus laevis)* (Hayes *et al.*, 2010). In the United States of America, the Environmental Protection Agency (EPA) has set the limit for atrazine in drinking water to be 3 µg/L (Kueseng *et al.*, 2009).

Figure 2.13 shows the application of atrazine to crops in South Africa in 2009, where the total use was over a thousand tons.

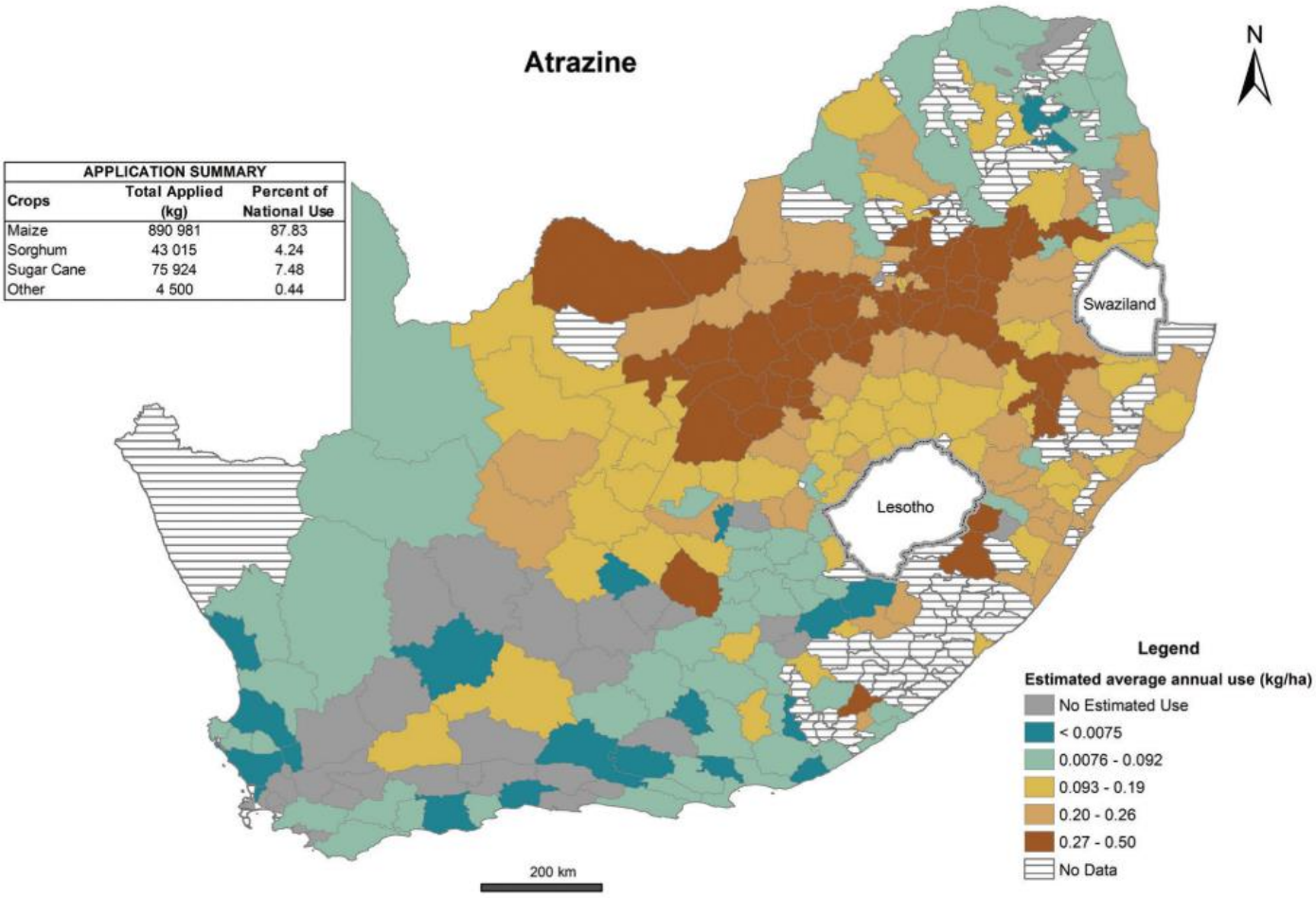


Figure 2.13: The average annual use of atrazine per hectare of agricultural land in magisterial districts of South Africa for the year 2009, estimated from pesticide sales and agricultural crop census data (Dabrowski, 2015)

Methods for trace triazine pesticide analysis, including atrazine, normally involve extraction and concentration steps prior to analysis. This is done by either liquid-liquid extraction, or solid phase extraction (SPE) using C18 sorbent (Ma *et al.,* 2003).

Terbuthylazine

Like atrazine, terbuthylazine (Figure 2.14) is also an organochlorine herbicide from the triazane chemical group (Jayaraj *et al.,* 2016), sometimes called chlorotriazine pesticides (Ferrer *et al.,* 2000), and is usually applied in a mixture with other triazine pesticides such as atrazine (MacBean, 2012). Terbuthylazine is applied as both a pre- and post-emergence herbicide (WHO, 2003c). Comparing the structure of terbuthylazine and atrazine shows that the two pesticides are very similar in chemical structure.

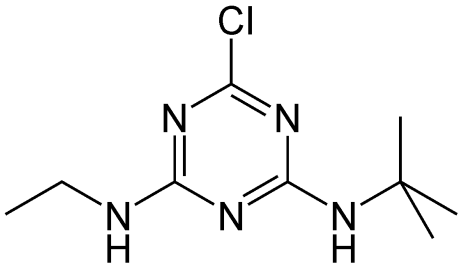


Figure 2.14: Chemical structure of terbuthylazine (NIST, 2014)

The MRL for terbuthylazine is 0.05 mg/kg for South African crops (Department of National Health and Population Development, 2012). The EU has similar legislation on terbuthylazine, where the MRL is set between 0.01 and 0.05 mg/kg depending on the produce, as published in the Official Journal of the European Union (EC No 149/2008).

Studies have been conducted demonstrating the effective action of terbuthylazine on chlorophyll-a in phytoplankton, stressing the long reaching environmental impact it can potentially have (Pereira *et al.,* 2017). There is no scientific evidence to suggest terbuthylazine is carcinogenic, however long term dietary studies in rats have observed lesions on the liver, lungs, thyroid and testes (WHO, 2003c).

In the WHO guidelines, first published in 1998 and later amended and adjusted in 2003, a health-based value of 7 µg/L was published for terbuthylazine in drinking-water (Papadopoulos *et al.,* 2009; WHO, 2003). High performance liquid chromatography with an ultraviolet detector (HPLC-UV) is generally used for terbuthylazine analysis with a LOD of 0.1 µg/L utilising a Water Oasis Mixed-mode cation exchange (MCX®) SPE cartridge for the extraction of terbuthylazine and its degradation products. Oasis MCX® sorbents act as a mixed-mode, strong cation-exchange, reversed-phase sorbent. A pre-treated sample of *Typha latifolia L.* wetland plant material was reconstituted with 1 N HCl solution before it was passed through the SPE cartridge. Subsequent analysis was performed utilising HPLC-UV (Papadopoulos *et al.*, 2009).

2.2.3.2: Chloroacetanilide pesticides

Chloroacetanilides are commonly used as herbicides (Anthony *et al.,* 2011). The chloroacetanilide group is derived from 2-chloroacetanilide with different methyl and ethyl groups attached to the benzene ring, an ether functional group is also present. Acetochlor and alachlor (chloroacetanilides) are herbicides used in agriculture for the control of broadleaf weeds and grasses mainly in corn, soybean, and sorghum fields (Yokley *et al.,* 2002).

Acetochlor

Acetochlor is generally used for the control of weeds in cornfields as a selective pre-emergence herbicide. Due to the extensive use of chloroacetamide herbicides, they can have a negative effects on the environment and human health due to their ability to cause nasal turbinate tumours (Wang *et al.*, 2015). The acetochlor chemical structure (Figure 2.15) contains a chlorinated acetamide functional group, and the aromatic ring has methyl and ethyl hydrocarbons attached in the ortho positions. An ethyl ether group is attached to the nitrogen in the acetamide group. Acetochlor is absorbed mainly by germinating plant shoots and secondly by roots, and inhibits protein synthesis (Lo *et al.,* 2008).

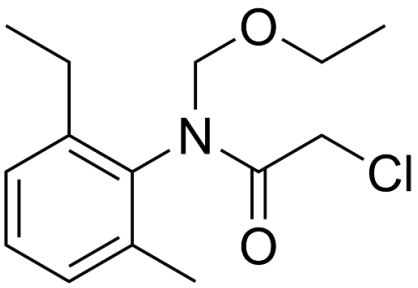


Figure 2.15: Chemical structure of acetochlor (NIST, 2014)

The MRL for acetochlor is 0.05 mg/kg for South African crops (Department of National Health and Population Development, 2012). The EU has legislation on acetochlor, where the MRL is set between 0.01 and 0.05 mg/kg depending on the produce, as published in the Official Journal of the European Union (Annex II to regulation (EC) No 603/2015).

In terms of human toxicity, no local or systemic signs of toxicity were observed in employees who handled acetochlor in laboratories or during the manufacturing process. Nor were any adverse effects reported in pesticide applicators (FAO/WHO Meeting on Pesticide Residues GENEVA, n.d.).

In literature a C18 SPE was used to extract several chloroacetanilide pesticides, including acetochlor, from water samples followed by subsequent HPLC-MS/MS analysis (Yokley *et al.*, 2002).

Alachlor

Alachlor is very similar in chemical structure to acetochlor (Figure 2.16). The structure contains a chlorinated acetamide functional group, and the aromatic ring has 2,6 diethyl hydrocarbons attached in the ortho positions. A methyl ether group is attached to the nitrogen in the acetamide group.

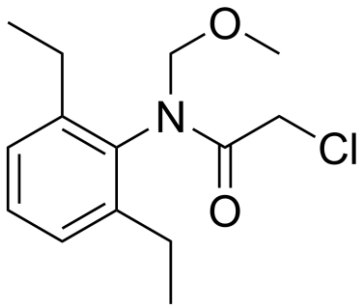


Figure 2.16: Chemical structure of alachlor (NIST, 2014)

The South African MRL for alachlor is 0.1-0.05 mg/kg depending on the crop type (Department of National Health and Population Development, 2012). The EU has similar legislation, where the MRL is set between 0.01 and 0.05 mg/kg depending on the produce, as published in the Official Journal of the European Union (Annex II to regulation (EC) No 899/2012).

Severe alachlor poisoning results in rather mild effects in most patients. A coma and hypotension rarely occurs in severe cases of oral ingestion that could cause fatalities (Lo *et al.,* 2008).

In literature a C18 SPE was used to extract several chloroacetanilide pesticides, including alachlor, from water samples followed by subsequent HPLC-MS/MS analysis (Yokley *et al.*, 2002).

2.2.4: Physicochemical properties of pesticides of interest

Octanol-water partition coefficient

The octanol-water partition coefficient (K_{OW}) is a measure of the hydrophilic- lipophilic partitioning of a compound. It is important for environmental studies, as it describes a pollutants affinity for water and how much the pollutant would partition into animals and soil. It is used in the determination of bio concentration factors of pollutants in aquatic life, and as such it is considered a required property in studies of new or hazardous chemicals (Cumming & Rücker, 2017; Dalrymple, 2005).

For this study, K_{OW} can be related to the affinity of an analyte to adsorb to the non-polar solid phase of a C18 SPE sorbent, rather than remain in an aqueous solution. Analytes with a low K_{OW} value (< 10) can be considered hydrophilic, leading to low bioaccumulation in the environment (Dalrymple, 2005). The higher the K_{OW} coefficient, the more hydrophobic an analyte becomes. For example a compound with a K_{OW} value of 10^4 will be very hydrophobic and tend to bio accumulate (Dalrymple, 2005). Log K_{OW} values a typically range from −3, indicating the compound was found to be very hydrophilic, to +10 meaning the compound was found to exhibit high hydrophobicity (Cumming & Rücke, 2017). Table 2.4 contains a summary of the log K_{OW} values for the four pesticides of interest in this study.

Table 2.4: The log K_{ow} and calculated K_{ow} values of the four selected pesticides

Pesticide	K_{OW}	Log K_{OW}	Reference
Atrazine	445.68 – 660.69 407.38	2.65- 2.82 2.61	(Dalrymple, 2005) (López-Roldán *et al.,* 2004)
Terbuthylazine	2511.88	3.4	(MacBean, 2012)
Acetochlor	1071.52	3.03	(López-Roldán *et al.*, 2004)
Alachlor	3311.32	3.52	(López-Roldán *et al.*, 2004)

Considering the log K_{OW} and K_{OW} values listed in the Table 2.4, all the selected pesticides are hydrophobic as a far higher concentration is expected to be found in octanol than water. Alachlor is the most hydrophobic, with the largest log K_{OW} value, terbuthylazine is thus slightly more hydrophobic then acetochlor with a larger log K_{OW}. Atrazine is the least hydrophobic of the four selected pesticides with the smallest log K_{OW}. This should in theory mirror the inverse elution order on a non-polar SPE sorbent, such as C18, with elution by a solvent gradient, starting from polar and moving to more non-polar. It is expected that atrazine will elute first and alachlor last.

Water solubility

For SPE, water solubility of the target analytes is also of interest since an aqueous solution is often used as a loading fraction. For extremely hydrophobic molecules, that are very insoluble in water, it might not be feasible to prepare an aqueous loading fraction as the target analyte will simply not

dissolve in a polar medium at the required concentration. Water might also be used to extract the target analytes from the sample matrix. Table 2.5 contains the water solubility of the analytes of interest, from which it is evident that alachlor and acetochlor are much more water soluble than atrazine and terbuthylazine.

Table 2.5: The water solubility of the pesticides of interest

Pesticide	Water solubility (µg/mL)	Reference
Atrazine	33	Štajnbaher & Zupančič-Kralj, 2003
Terbuthylazine	8.5	WHO, 2003b
Acetochlor	223	US EPA, 2006
Alachlor	242	WHO, 2003a

2.3 Cannabis use in South Africa

Cannabis refers to the plants in the genus *Cannabis* and family *Cannabaceae*, which includes various species or sub-species such as *C.sativa, C.indica* and *C.ruderalis* which are all widely referred to by the same common name, cannabis. The plant, or parts or products thereof, along with tetrahydrocannabinol (THC), the psychoactive substance that gives a user a "high", are currently listed as Schedule 7 substances in terms of the Medicines and Related Substances (South African Health Regulatory Authority, 2019). An exception is made for tetrahydrocannabinol present in processed hemp (*C.indica*) fibre and products containing not more than 0,1% of THC in a form not suitable for ingestion, smoking or inhaling. Another exception is made for processed products made from cannabis seed containing not more than 0,001% of THC. Many countries also make an exception when cannabis is used for medicinal purposes. Marijuana and hemp are colloquial terms often used to describe subspecies or strains of the family *Cannabaceae*. The plant can be cultivated to contain different ratios and concentrations of cannabinoids. The high THC-containing cannabis plant (*C.sativa*) is generally utilised for recreational purposes and is commonly referred to as marijuana, weed, ganja or insangu. Due to extensive cross breeding and the many different strains now available, the original strains are almost impossible to find in unadulterated form and THC quantification is necessary to indicate if the plant can be classified as hemp (Brian & Mahmoud, 2016).

The map in Figure 2.17 was taken from a book:" The Analytical Chemistry of Cannabis" published in 2016, showing cannabi*s* as decriminalised in South Africa, although this was before any official amendment was made to the status of cannabis legality in South Africa.

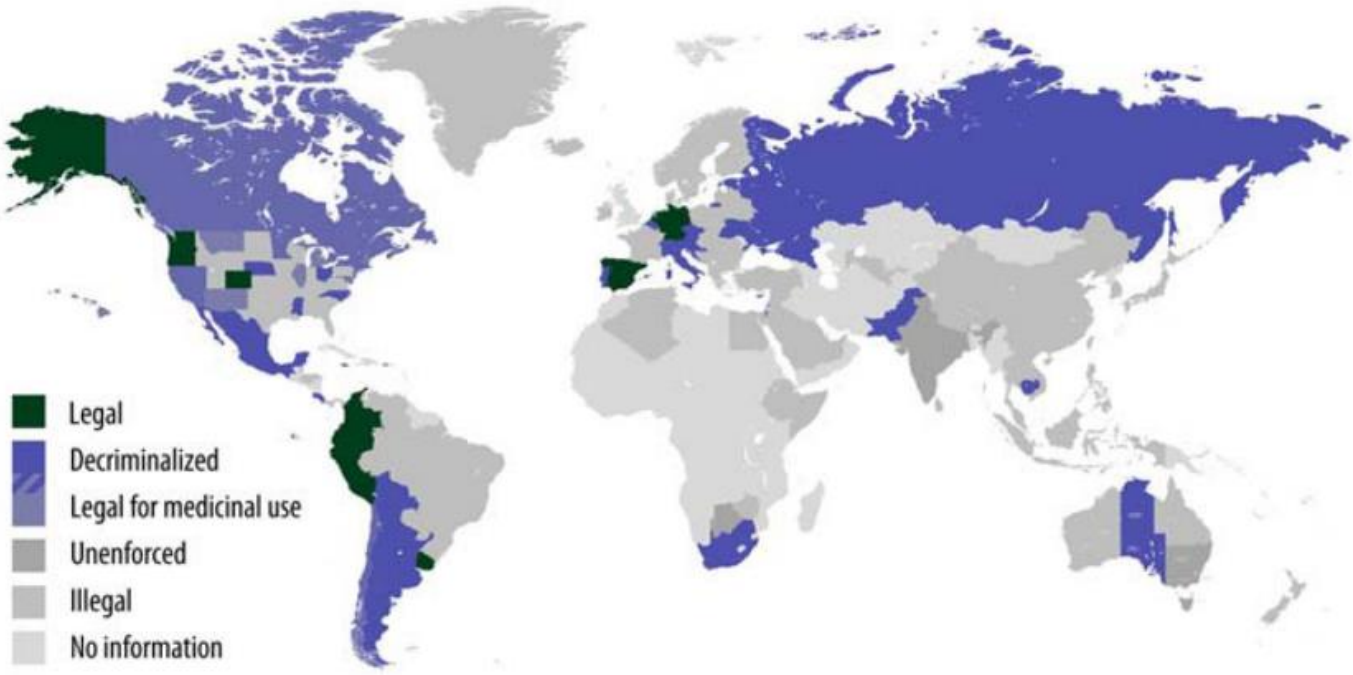


Figure 2.17: Global variation in the legality of cannabis use as of 2015. The legal status for cannabis is continuously changing (Brian & Mahmoud, 2016)

In 2017 it became possible to apply for a cultivation licence for medicinal cannabis from the Medicines Control Council (MCC). The document released by the MCC entitled ”Cultivation of cannabis and manufacture of cannabis-related pharmaceutical products for medicinal and research purposes” outlines in great detail how and when medicinal cannabis is allowed to be cultivated and how the processing of the plants should take place (Gouws, 2017), and from this time there was an increase in the demand for analysis of cannabis and cannabis related samples by commercial laboratories although it was still not legal to sell or use cannabis in South Africa.

On 18 September 2018, the Constitutional Court handed down a judgment in Cape Town that allowed an adult person to use, possess and cultivate cannabis in private for his or her personal consumption, whilst using cannabis in public or in the presence of children or a non-consenting adult person is still illegal (Nkambule *el al.*, 2019).

Cannabidiol (CBD) is listed as a schedule 4 substance by the Department of Health of the South African Government (Department of Health, 2020; Motsoaledi, 2019). CBD-containing preparations were exempted from prosecution by the Minister of Health for a limited period until May 2020, as per an exclusion notice published in Government Gazette No. 42477 on 23 May 2019. Anyone who imports or manufactures a CBD-containing medicine must now be in possession of a licence issued in terms of Section 22C(1)(b) of the Medicines Act and comply with any relevant standards, including current good manufacturing practice standards (Motsoaledi, 2019). Manufacturers must be able to present verified

analytical results by an accredited laboratory of the CBD and THC content of the manufactured products when requested (Motsoaledi, 2019; Nkambule *et al.,* 2019).

On 4 November 2019, the South African Police Service (SAPS) and South African Health Products Regulatory Authority (SAHPRA, the reformed MCC), released a joint media statement that warned that dealing in cannabis and cannabis related products is still illegal except where specifically allowed in terms of the Medicines and Related Substances Act. In other words, only 0.001% THC may be present in products. From the statement it can be deduced that some of the illegal businesses were posing to be operating legally in terms of the Traditional Health Practitioners Act (No. 22 of 2007). According the aforementioned act, traditional medicine is defined as: "An object or substance used in traditional health practice for the diagnosis, treatment or prevention of a physical or mental illness or any curative or therapeutic purpose, including the maintenance or restoration of physical or mental health or well-being in human beings but does not include a dependence-producing or dangerous substance or drug" (National Department of Health South Africa, 2008). The released joint statement, however, made it clear that the Traditional Health Practitioners Act does not create a mechanism to sell cannabis products that are not exempted in terms in the Medicines Act. Dealing in cannabis remains a serious criminal offence in terms of the Drugs and Drug Trafficking Act (No 140 of 1992) (Nkambule *et al.,* 2019).

SAHPRA is tasked with regulating (monitoring, evaluating, investigating, inspecting and registering) all health products and clinical trials in South Africa. Health products include complementary medicines, medical devices, and *in vitro* diagnostics. SAHPRA's mandate is outlined in the Medicines and Related Substances Act (Act 101 of 1965 as amended) as well as the Hazardous Substances Act (Act No. 15 of 1973) (Nkambule *et al.,* 2019).

On 22 May 2020, a Government Gazette (Vol. 659, No. 43347) announced amendments to the schedules for CBD and THC. CBD was listed as a Schedule 4 substance and under two conditions a Schedule 0. The first condition relates to complementary medicines, containing no more than 600 mg CBD per sales pack, and providing a maximum daily dose of 20 mg of CBD. The second condition refers to processed products made from cannabis plant material containing no more than 0,0075% CBD, where only the naturally occurring quantity of cannabinoids found in the source material are contained in the product. THC is now listed as a Schedule 6 substance, except for products made from hemp, not intended for consumption, that contain under 0.2% THC, or processed products made from cannabis intended for consumption that contain no more than 0.001% THC. The Gazette also states that THC is exempt when raw plant material is cultivated, possessed, and consumed by an adult, in private, for personal consumption. By removing cannabis (THC) from Schedule 7, the requirement of

a permit for the manufacture of a Schedule 6 product is in accordance with South Africa's obligations as a signatory to the 1961 Single Convention on Narcotic Drugs (Brian & Mahmoud, 2016; Gouws, 2017). Cannabis is thus unregulated and unscheduled if it is cultivated for personal consumption, however for production and sale a permit is required from SAHPRA.

2.3.1: Cannabis samples submitted for analysis

Commercial analytical laboratories in South Africa generally receive three types of cannabis samples namely plant material, infused oils and full extract cannabis oil (FECO), refer to Figure 2.18, whilst many other different infusions and edibles are also analysed upon request. Further refined cannabis products include CBD isolates, where CBD is extracted and purified using chromatographic techniques until a white crystalline powder is obtained. Supercritical fluid extractions, typically using supercritical CO_2, are also employed for the extraction of cannabinoids and terpenes from plants.

The plant material can vary in quality and dryness. Most growers source the buds and flowers that contain the most cannabinoids, while others choose to pulverise the entire plant, resulting in a mixture of leaves, flowers and stems that generally contain a much lower concentration of cannabinoids but leaving a far larger sample for extraction. Growers can also choose to separate male and female plants, which causes the plant to continue flowering and never produce seeds, increasing the harvestable flowers of each plant and resulting in an overall higher cannabinoid concentration (Brian & Mahmoud, 2016).

Infused oils are produced when the cannabis plant material is placed in oil and heated to extract the cannabinoids. The resulting oil is then filtered to remove the excess plant material. The types of oil used are usually various plant-based oils such as grape seed, coconut, hempseed, olive and avocado oils. Medium-chain triglyceride (MCT) oils or propylene glycol are also often used, especially when a vape oil is produced. The full extract cannabis oil (FECO) involves the use of solvents, such as ethanol or isopropanol to extract the cannabinoids. The solvent is then evaporated leaving a sticky substance with concentrated cannabinoids. The FECO sample matrix is of the greatest concern in terms of safety issues, since the extraction is not selective enough to only extract cannabinoids. Thus any contaminant that is soluble in the selected solvent will also be concentrated in the final product. The sample matrix is also "dirtiest" of all the discussed sample types, as many contaminants and matrix analytes are concentrated in the FECO.

Figure 2.18: Examples of FECO (a), infused oil (b), CO_2 extract (c) and isolate CBD (d) samples

2.3.2: Cannabis maximum residue limits for the four selected pesticides

According to the regulations governing MRLs for atrazine, alachlor, acetochlor and terbuthylazine, there should be no more than 0.05 mg/kg (0.05 µg/g) in South African crops (Department of National Health and Population Development, 2012). In Canada, specific limits apply for different cannabis products, and the limits are generally set out as follows: 3.0, 2.5 and 1.5 µg/g for dried cannabis flowers, oil and fresh plant material respectively (Atapattu and Johnson, 2020). The higher limits are justified by the amount of product used by the end user, as it is expected that less dried flower will be consumed in comparison to fresh plant material (Atapattu and Johnson, 2020). It should however be noted that neither atrazine and terbuthylazine or acetochlor and alachlor are mentioned in the Canadian monograph.

The major goal of SPE is to pre-concentrate and purify the pesticides before analysis. If 20 mg of sample material is weighed, and a concentration of 0.05 mg/kg (50 µg/kg) is present, then

$$0.05\frac{mg}{kg} \times 0.000020\ kg$$

$$= 0.000001\ mg$$

$$= 0.001\ \mu g$$

This means that a total of 0.001 µg atrazine is present in a 20 mg sample at the MRL. If this can be pre-concentrated to 50 µL by SPE and vacuum concentration, then the final concentration is 0.02 µg/mL. This is much too low for GC-MS analysis and more sample is thus required. Table 2.6 contains a summary where this calculation was performed on different sample amounts at the 0.05 mg/kg MRL to determine the theoretical final concentrations of the extracted pesticides.

Table 2.6: Sample amount vs final concentration of pesticides in the sample extract at the 0.05 mg/kg MRL

Sample amount (mg)	Pesticide present (µg) at 0.05 µg/g MRL	Concentration of pesticide in a sample of 50 µL for 0.05 ug/g MRL (µg/mL)
20	0.001	0.02
100	0.005	0.1
1000	0.05	1
3000	0.15	3
10 000	0.5	10

Unfortunately, a 10 g sample of cannabis flower is a very large amount. Of course, this calculation assumes 100% of the pesticide can be recovered, which is impossible. From Table 2.6, it is clear that at least one gram of plant material would be required to allow for detection of the selected pesticides at the South African crop general limit with a final volume of 50 µL. This is based on the assumption that 1 µg/mL of pesticide can be detected on the GC-MS in the sample matrix with a signal to noise ratio of at least 10. The LOQ of the instrument was determined in Section 3.9.2.

2.4: Pesticide extraction and sample preparation

Sample preparation methods involve an organic solvent extraction step, to remove the pesticide from the sample. Solvents used for pesticide extraction include methanol, ethyl acetate, acetone and acetonitrile (Zhang *et al.,* 2011).

A clean-up step is then required to remove matrix co-extractants from the sample prior to analysis. The co-extractants can cause matrix effects and interferences during pesticide analysis (Zhang *et al.*, 2011). A pre-concentration step is then required to increase the concentration of the target analytes (Wang *et al.*, 2015). The following sections discuss and explain the many extraction methods associated with pesticide residue analysis from plant material.

2.4.1: Soxhlet extraction

Soxhlet extraction has been demonstrated to be effective for extraction of residual pesticides from a variety of matrices including food and soil (Lang *et al.,* 2005). There are also several literature examples that use Soxhlet extraction to remove the template molecule from the MIP after synthesis (Chen *et al.*, 2014; Ferrer *et al.*, 2000; Kueseng *et al.*, 2009).

Soxhlet extractions can be run for a very long time, at least a couple of hours to a full day, and the sample is continuously washed with solvent. Any volatile solvent can be used, such as acetonitrile, methanol or ethanol. The condenser can also be coupled to a chiller, to allow for the use of more volatile solvents, such as hexane. The final extract can be concentrated by evaporation of the solvent under vacuum or heat, depending on the target analytes (Patnaik, 2004).

2.4.2: Microwave-assisted extraction

Since the 1990s, great efforts have been put into developing new and better ways to reduce laboratory-generated waste, while also improving the detection limits of analytical methods. Many extraction methods, such as Soxhlet, begin with submerging a desired amount of sample or homogenised subsample in a rather large volume of organic solvent. A minute portion of this solvent is carried through the entirety of the analytical process; the extraction process is thus quite wasteful (Pylypiw *et al.,* 1997). In contrast, microwave-assisted extraction (MAE) can use up to 90% less solvent in the extraction process, and is a lot less time consuming, making it suitable for routine analysis. As the name suggests, microwave energy is used to heat up samples and their associated solvents in closed and pressurized containers. MAE has been demonstrated to be effective for several crops and pesticide classes (Pylypiw *et al.*, 1997). An obvious limitation is that the pesticides to be analysed cannot be temperate sensitive, unless their breakdown products are well known. The optimal temperature for pesticide extraction is also affected by the crop matrix. MAE parameters such as

temperature, pressure and time need to be optimized on an individual pesticide and matrix basis (Pylypiw *et al.*, 1997). MAE is reliable and rapid and is therefore ideal for routine analysis of a wide variety of pollutants. The only downside is the cost of the instrumentation required, as it is very expensive when compared to other extraction methods (Wan *et al.,* 2010).

2.4.3: Quick, easy, cheap, effective, rugged, and safe (QuEChERS) method

The quick, easy, cheap, effective, rugged, and safe (QuEChERS) method is used worldwide in many accredited laboratories on a routine basis, due to the advantages evident in the method name. It was first introduced in 2003 by Anastassiades and Lehotay (Anastassiades *et al.,* 2003) where acetonitrile extraction was followed by dispersive solid-phase extraction for the determination of pesticide residues on fruits and vegetables, and recoveries were reported to be between 85 and 101% with an RSD of <5%. The method did not actually include any new concepts, as solid-liquid extraction with salting out and dispersive SPE were already well known preparation procedures. However, the combination of solvents, salts and sorbents reported were found to be so effective that the method has since become standard practice worldwide. Many vendors sell QuEChERS kits, with pre-packed salts and sorbents to further quicken the method. There are now many variations of the method depending on the application and analytes being extracted (Anastassiades *et al.*, 2003; González-Curbelo *et al.,* 2015).

Acetonitrile is the most popular QuEChERS solvent to extract pesticide residues from samples (Zhang *et al.*, 2011). Salt is used in conjunction with acetonitrile and causes phase separation between the aqueous and acetonitrile solvents. The employed salts may include anhydrous magnesium sulphate, sodium chloride or sodium acetate. A dispersive solid phase extraction is then performed on the acetonitrile extract with a mixture of magnesium sulphate and primary-secondary amine (PSA) sorbent which removes many polar components from the matrix such as organic acids, certain polar pigments, and sugars. Depending on the specific application, additional clean-up steps or reagents can be included, such as other dispersive sorbents (C18 or graphitized carbon black), and solid phase extraction cartridges can even be employed. The extract may also be diluted prior to LC-MS/MS analysis, minimizing matrix effects, at the cost of reduced sensitivity (Zhang *et al.*, 2011). A large array of fruits and vegetables have been analysed for 209 different pesticide residues after QuEChERS extraction, including carbamates, organophosphates, phenylureas, anilides, benzoyl phenylureas, conazoles, macrocyclic lactone, neonicotinoids, strobilurines and triazines (Zhang *et al.*, 2011).

2.4.4: Ultrasound assisted solvent extraction

Ultrasound assisted extraction (UAE) was a key development to achieve sustainable greener chemistry extractions (Chemat *et al.,* 2017). The following mechanisms are associated with ultrasound-assisted extraction: fragmentation, erosion, sonocapillary effect, detexturation, shear stress, and sonoporation. When sonicating a liquid medium containing a solid, for example a compressed pill or a plant sample, fragmentation can often be observed where the sample breaks up into fine pieces or the plant cells break apart. Scanning electron microscopy (SEM) images of samples subjected to UAE indicate erosion on the surface (Khadhraoui *et al.,* 2018). Erosion is particularly observed in plant samples where the top layer of cells is lysed and fine hairs, follicles and trichomes break apart. The ultrasonic capillary effect, or sonocapillary effect, involves the increased penetration depth and velocity of the solvent into canals and pores under sonication. Sonoporation refers to the permeation of a cell membrane under ultrasonic conditions. Sonoporation has been used in the field of biology for *in vitro* cell uptake of drugs or genes through the cell membrane under high ultrasound frequencies > 500 kHz. For extraction purposes, some cellular content can pass through the cell membrane into the extractive solvent. During UAE of a solid-liquid mixture, shear forces are generated within the liquid around the solid material. Shear forces, or turbulences, are caused by oscillation and collapse of cavitation bubbles within the liquid. This oscillation effect is important when considering mixing or emulsification of two immiscible fluids. Detexturation, sometimes called destruction, is most often observed in plant cells, causing the texture of the sample surface to change during ultrasound extraction. For plant-based samples, this means the complete destruction of the protective cuticle film covering the epidermis of leaves. Figure 2.19 shows a visual representation of these processes along with SEM images of the surface of the plant material (Chemat *et al.*, 2017; Khadhraoui *et al.*, 2018). UAE forms an integral part when analysing plant-based samples in order to ensure an effective extraction is achieved.

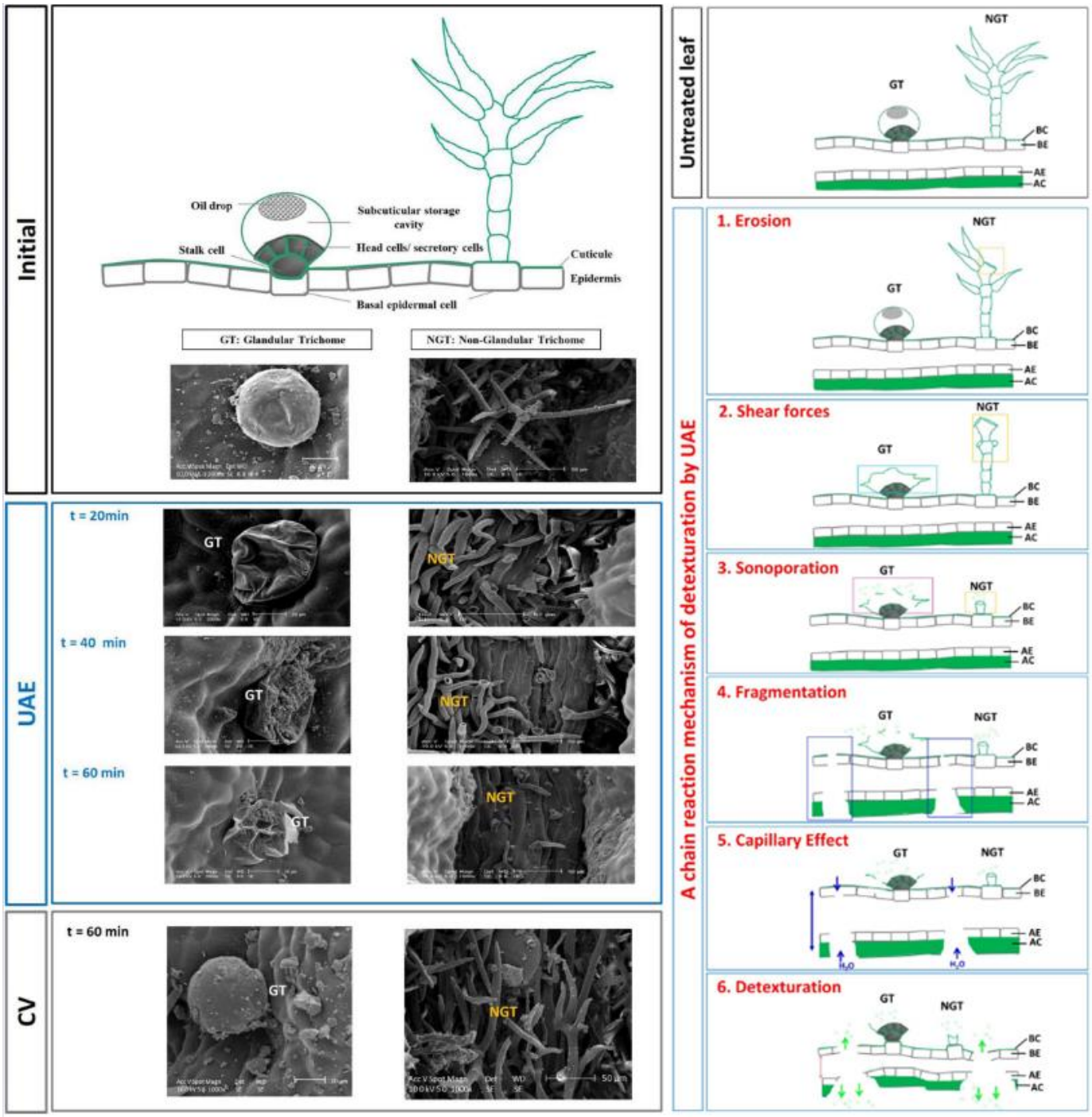


Figure 2.19: Visual representation of the mechanisms associated with UAE alongside the SEM images visualizing the effect thereof on the plant material sample (Khadhraoui et al., 2018) *GT: glandular trichomes, NGT: non- glandular trichomes, BC revers to the basal cuticula and BE epidermal cell layer. AC: adaxial cuticle; AE: adaxial epidermal cells. The conventional solvent (CV) SEM images are for comparison to UAE, and were done for 60 min.*

2.4.5: Liquid-Liquid extraction

A commonly used and widely applicable sample clean-up technique is liquid-liquid extraction (LLE). Methods for the extraction of analytes from water exploit the partitioning of analytes between the aqueous phase and a water-immiscible solvent (Headley *et al.*, 2010). LLE is still regarded as a universal extraction method for screening purposes (Anthony *et al.,* 2011) and has been recently used for the extraction and analysis of pesticides, such as paraquat and glyphosate, from cannabis related products (Atapattu and Johnson, 2020).

LLE techniques have the serious disadvantage of using large volumes of solvents. Nevertheless, LLE continues to be used in many laboratories, because it is both cost and time effective (Headley *et al.*, 2010).

2.4.6: Solid phase extraction

Solid phase extraction (SPE) is widely used for the extraction and pre-concentration of many pesticides in a multitude of environmental, food and biological samples (Wang *et al.*, 2015). SPE was developed with the aim of reducing solvent consumption during sample clean-up (Pylypiw *et al.*, 1997). It exploits the partitioning of analytes between a liquid phase, usually aqueous, and the sorbent (Headley *et al.*, 2010), where dissolved analytes are separated from other compounds in the sample matrix based on chemical properties such as polarity or pKa value.

The sample is passed through the SPE cartridge and the compounds in the sample that have affinity for the stationary phase or sorbent are retained on the cartridge while the rest of the compounds pass through. The solution that passes though the SPE can either be collected or discarded depending on whether the desired analyte has been trapped on the SPE or if the undesired compounds have been removed from the solution. If the analytes are trapped on the sorbent, they can be removed from the sorbent by a solvent that the analyte has a good affinity for, also known as the eluent (Hennion, 1999).

Classically a C18 SPE cartridge is used for the extraction of azine pesticides (Zhu *et al.*, 2005). Conventional SPE sorbents are silica with bonded functional groups such as C8 and C18, which provide selectivity for non-polar compounds (Figure 2.20). Anion and cation exchange sorbents are commonly employed for analytes that have a protonatable or de-protonatable functional group such as an amine or carboxylic acid. For a strong anion exchange sorbent, the stationary phase is a quaternary amine, thus giving selectivity for anions since the sorbent is positively charged (Figure 2.22). The lack of analyte selectivity of these materials usually leads to the co-extraction of several matrix components with similar polarities or pKa values that can interfere in the analysis of target analytes (Wang *et al.*, 2015).

A typical SPE method contains five steps: first the cartridge is rinsed with a non-polar or slightly polar solvent; this is referred to as wetting or pre-treatment. Secondly, a buffer solution or solvent with the same composition as the sample is passed through the column to condition the silica surface. The sample is then passed through the column and the analytes are trapped on the stationary phase. The column is subsequently washed with a solvent that has a low affinity for the analyte, thus removing impurities but not affecting the analyte on the solid phase. Lastly, the analyte is removed from the column using an eluent. The goal is to obtain extracts free from matrix interferences in as few steps as possible and this is optimised in the development of a SPE procedure (Hennion, 1999; Sigma-Aldrich, 1998).

SPE cartridges come in many shapes and sizes, where the syringe-barrel and cartridge types are the most popular (Hennion, 1999). The syringe shaped cartridge was used in this study. To limit the amount of leaching that can take place, manufacturers used medical-grade polypropylene or polyethylene for the body of the SPE cartridge. Limitations of the conventional syringe shaped SPE cartridge include the low flow rate and the blocking of the top frit, especially if the sample contains sediment or insoluble particles. This means that large samples, 500 mL for example, will take a very long time to pass through the SPE cartridge and the sample needs to be carefully filtered. There are SPE cartridges available with built in polytetrafluoroethylene (PTFE) filters, to help speed up this process. Carbon black filters are also a popular option (Hennion, 1999), but may incur loss of analytes. The less popular disk shaped SPE cartridge allows for a faster flow rate without causing channelling, due to the larger cross-sectional area and thin sorbent bed, although the disk SPE can still block if there are suspended particles in the sample (Hennion, 1999). Table 2.7 contains a summary of the types of SPE sorbents commonly used.

Table 2.7: Sorbents commonly associated with SPE and the respective target analytes for which they are used (Patnaik, 2004)

Sorbent	Description	Polarity or classification of target compounds
C8	Octyl bonded silica	Nonpolar compounds
C18	Octadecyl bonded silica	Nonpolar compounds
C-phenyl	Phenyl bonded silica	Nonpolar or phenolic compounds
C-CN	Cyanopropyl bonded silica	Moderately polar to polar compounds, carbohydrates and cations
Si	Silica gel	Polar compounds
C-diol	Diol bonded silica	Polar compounds
Florisil	Magnesium silicate	Polar compounds
C-NH_2	Aminopropyl bonded silica	Carbohydrates, weak anions, organic acids
C-SCX	Sulfonic acid bonded silica (strong cation exchanger)	Strong cations, organic bases
C-SAX	Quaternary amine bonded silica (strong anion exchanger)	Strong anions, organic acids
C-WCX	Weak cation exchanger	Weak cations

It is reported in literature that sub-parts per trillion concentrations of atrazine have been analysed in aqueous solution using GC-MS after C18 SPE. The cartridge was washed beforehand using ethyl-acetate, methanol and water, and then eluted with ethyl acetate (Ma *et al.,* 2003).

With regards to the C18 SPE sorbent (Figure 2.21 and Figure 2.22) analytes are separated from the matrix in terms of their polarity. This means the polarity of the loading and elution fractions should be changed accordingly depending on the goal of the SPE process. The polarity is changed by using different solvents or mixtures thereof as loading, wash and elution solvents. There are two types of C18 SPE sorbents commonly used, namely C18-U (uncapped siloxane group, Figure 2.21) and C18-E (end-capped siloxane group, Figure 2.20). The C18-E sorbent was used in this study. The C18-U has secondary polar interactions with the analytes because of the active silanol group giving the sorbent moderate hydrophobic selectivity and slight polar selectivity, whereas the C18-E only has selectivity based on hydrophobicity. For the SAX SPE sorbent (Figure 2.22), the analytes are separated from the matrix according to their pKa values, and the acidity of the loading and elution fractions are thus important. The C8 and C18 sorbents have the disadvantage of not being able to selectively target analytes, which usually leads to the co-extraction of several matrix components that can interfere in the analysis of target pesticides (Wang *et al.*, 2015).

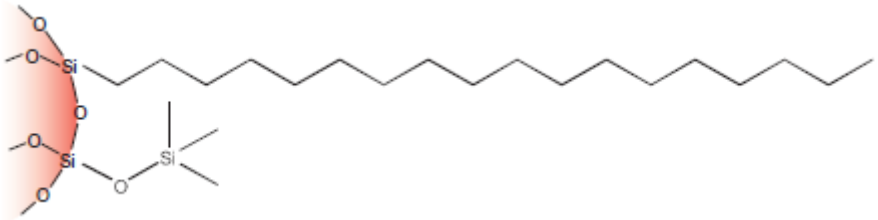


Figure 2.20: The chemical structure of C18-E SPE sorbent, with an end capped trimethyl silane group (Supelco; Sigma-Aldrich, 1998)

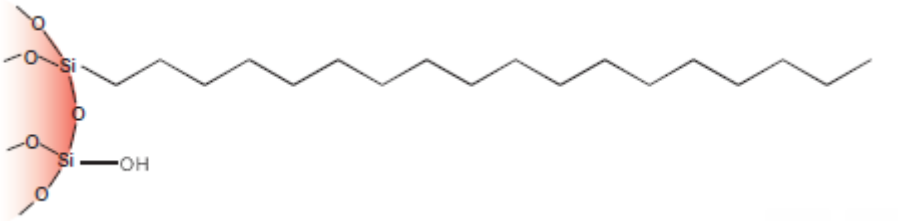


Figure 2.21: The chemical structure of C18-U SPE sorbent, with an uncapped silanol group (Supelco; Sigma-Aldrich, 1998)

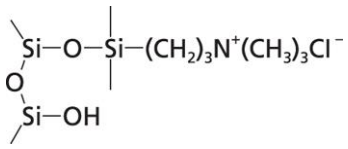


Figure 2.22: The chemical structure of a SAX (strong anion exchange) SPE sorbent (Supelco; Sigma-Aldrich, 1998)

A relatively new and growing trend in SPE is the use of molecularly imprinted polymers (MIPs) as sorbents. The advantages of utilising MIPs as SPE sorbents include their high selectivity, high affinity constants and good stability (Headley *et al.*, 2010). MIPs are discussed in detail in Section 2.7.

2.4.6.1 Solid phase microextraction

Solid phase microextraction (SPME) utilises a fibre coved with a sorbent. The sorbent can be liquid or solid. SPME has been used for the extraction of organochlorine pesticides from liquid and gas matrices as it preconcentrates target analytes. SPME does not use any solvents, and can be applied for the extraction of volatile and semi volatile analytes as well as nonpolar and polar compounds (Gondo *et al.,* 2016).

SPME allows for minimal sample preparation steps and pre concentrates the sample from a variety of matrices including water, soil, plant materials, milk, vegetables, fruits, medicinal plants infusions and tea infusions (Gondo *et al.*, 2016).

SPME has been used for the extraction of a series of organochlorine pesticides from plant material, including roots, stems and leaves. LODs were reported to be 0.1 µg/L in the extracts analysed utilising GC- time of flight MS (Obuseng *et al.,* 2013).

2.4.7: Extraction and analysis of pesticides from cannabis

Medical cannabis has become an important sample matrix in North America and Canada with recent decriminalisation in different states (Moulins *et al.*, 2018). Pesticide analysis of cannabis samples typically involves acetonitrile extraction followed by solid phase extraction clean-up and subsequent analysis with HPLC-MS/MS, GC-MS/MS or GC-MS (Moulins *et al.*, 2018). A recent review article found dispersive solid phase extraction (dSPE) and modified QuEChERS to be the most prevalent methods for pesticide extraction from cannabis samples (Taylor & Birkett, 2020).

Application notes are readily available from instrument manufactures dealing with the analysis of pesticides on cannabis; the following literature example is from such a PerkinElmer application note. It was reported that all 59 pesticides on the Oregon list were analysed utilising QuEChERS and LC-MS/MS for sample preparation and analysis, respectively. The preparation method utilised a modified QuEChERS method with both C18 and PSA dispersive SPE (dSPE) sorbents (Armstrong & Carnagey, 2017).

Another study used C18 SPE cartridges for the extraction of pesticides from both cannabis flowers and leaves. For oil extracts an Enhanced Matrix Removal (EMR)-Lipid tube was used. Both HPLC-MS/MS and GC-MS were required for the analysis of the 40 target pesticides (Moulins *et al.*, 2018).

A recent review article on pesticide analysis of cannabis and cannabis products reports preparation methods that include liquid-liquid extraction, solid- phase extraction, solid-phase microextraction and QuEChERS. Analysis of the extracts involves separation techniques such as thin-layer chromatography, capillary electrophoresis, HPLC and GC in combination with ultraviolet and MS detectors (Atapattu and Johnson, 2020), as discussed in more detail in Sections 2.5 and 2.6.

2.5: Chromatographic techniques for the analysis of pesticides

Over 850 active substances are used as pesticides worldwide, and there is currently no single technique that can analyse all these pesticides (Alder *et al.,* 2006). Many pesticides are acidic, but some are basic. Pesticides may contain halogens and others phosphorus, sulphur and/or nitrogen or even metals. Some pesticides are very volatile, but several do not evaporate at all. This diversity causes serious problems in the development of a universal analytical method (Anthony *et al.,* 2011). GC and LC coupled to MS are the most powerful analytical instruments currently available for residual pesticide analysis (Kwon *et al.,* 2012). GC-MS and LC-MS/MS are considered standard methods for

quantitation of chloroacetamide herbicides (Wang *et al.*, 2015). For more than 20 years now, gas and liquid chromatography have been well established methods for the analysis of herbicides, and SPE is commonly used as a pre-concentration or purifying step prior to analysis (Matsui *et al.,* 1997).

GC-MS is extensively used for multiresidue pesticide analysis, as the MS provides more selectivity than other detectors associated with GC (Alder *et al.,* 2006). The extensive use of GC-MS in pesticide analysis is seen in analytical manuals (Anthony *et al.,* 2011), application notes from instrument producers, and in many journal publications according to a literature review article (Alder *et al.,* 2006). GC-MS is suitable for many organochlorine, organophosphorus, pyrethroid and other volatile and thermally stable pesticides (Zhang *et al.*, 2011). However, some thermally unstable, very polar or non-volatile pesticides cannot be analysed by GC methods, thus LC might be employed for the analysis of such pesticides (Zhang *et al.*, 2011).

Methods based on LC with ultraviolet (UV) diode array detection are not common, as this method is less selective therefore complex sample matrix or multiresidue analysis is challenging. With the extensive development of MS in recent years, particularly with electrospray and atmospheric pressure chemical ionization for LC and HPLC compatibility, MS has become common place for pesticide analysis. Tandem MS coupled with LC (LC-MS/MS), has overcome many sample clean-up and selectivity problems associated with complex sample matrices (Alder *et al.,* 2006). LC-MS/MS has been gradually gaining popularity for multiresidue pesticide analysis, as it has superior selectivity and sensitivity compared to GC-MS (Zhang *et al.*, 2011). A review of 500 high priority pesticides has demonstrated that LC-MS/MS is superior to GC-MS in terms of LOQs and the limiting dwell time of the single quadruple GC-MS that only allows for a maximum of 25 characteristic ions to be monitored at a time. LC-MS/MS thus has a wider scope, and increased sensitivity and selectivity when compared to GC-MS (Alder *et al.,* 2006). A literature review on pesticide analysis, specifically with respect to the cannabis sample matrix, however found that both HPLC-MS/MS and GC-MS are extensively used for the analysis of a wide range of pesticides (Taylor & Birkett, 2020).

MS is discussed in greater detail in Section 2.6, whilst the use of GC and LC in pesticide analysis is discussed in greater detail in the following sections.

2.5.1: Gas chromatography

Substances that have a vapour pressure of at least 60 torr may be amenable to GC. GC columns can typically be heated to 350 °C limiting the analytes, or derivatives of the analytes, that can be analysed, as they must be volatile at the column temperature (Patnaik, 2004).

There are many examples of pesticide analysis in the literature utilising GC-MS or GC-MS/MS, as summarised in Table 2.8 for the selected target pesticides in this study. A reference is made to the column and detector used in the GC analysis and the sample matrix the pesticide was extracted from. Only pesticides of interest in this study are reported although it should be noted that many of the referenced articles focussed on more pesticides than the ones mentioned in Table 2.8.

Table 2.8: Examples of the four selected pesticides analysed utilising GC with different detectors and column phases

Pesticide(s) of interest	Sample matrix	Clean-up technique	GC- Column	Detector	Reference
Atrazine, alachlor and terbuthylazine	Fruit	Modified QuEChERS- graphene carbon black and C18	5 MS	MS/MS	Fochi *et al.,* 2010
Atrazine	Food Crops	QuEChERS- PSA and C18	Restek Rxi-5 MS	Time of flight MS	Kwon *et al.,* 2012
Atrazine	Water and soil	MISPE	Agilent DB-5	Nitrogen Phosphorous Detector	Zhu *et al.,* 2005
Atrazine	Tomato	QuEChERS- PSA and C18 sorbents	Agilent J&W HP-5 MS	MS	Zou & Zhai, 2015
Atrazine, terbuthylazine and alachlor	River water	MISPE	Restek Rtx-5MS	MS/MS Ion-Trap	Guzzella *et al.,* 2008
Atrazine, alachlor	Fruit and vegetables	DCM extraction and filtration-	Restek Rtx-5MS	MS/MS Ion-Trap	Schachterle & Feigel, 1996
Atrazine	Fruit and vegetables	Direct sample introduction	Folsom DB 5 MS	MS/MS	Lehotay, 2000

From Table 2.8, the 5% biphenyl (DB-5 MS and Rtx-5MS) stationary phase is clearly a very popular choice for the analysis of the selected pesticides by GC-MS.

2.5.2: Liquid chromatography

Chromatographic separation in liquid chromatography (LC) can generally be divided into two classes, either high-performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC). LC is initiated by interaction of the analytes with both the stationary and mobile phases and is based on the compound's affinity for each of these phases. The difference between HPLC and UHPLC is that the pressure generated by the solvent pump is much higher for UHPLC, thus it is sometimes referred to as ultra-high pressure liquid chromatography. The higher pressure results from the packing and particle size of the stationary phase of the column used for UHPLC, with stationary phase particles < 2 µm. The higher pressure and smaller internal volume results

in quicker runs and faster analyte separation. Both the solid and mobile phases can be adjusted allowing for a wide range of selectivity and applications. The mobile phase polarity can be altered by choosing a different solvent, or running a solvent gradient, where two or more solvents are gradually mixed over the course of the sample run (Patnaik, 2004).

There are many examples of pesticide analysis in literature utilising LC-MS or LC-MS/MS. Table 2.9 contains a summary of selected studies based on the analysis of the selected pesticides of interest in this study. A reference is made to the column, mobile phase and detector used in the LC analysis and the sample matrix the pesticides were extracted from. Although only the pesticides selected for this study are reported, many of the methods focused on more than the mentioned pesticides.

Table 2.9: Examples of the four selected pesticides analysed in different sample matrixes utilising LC with different detectors and columns

Pesticide(s) of interest analytes	Sample matrix	Clean-up technique	LC column	Mobile phase	Detector	Reference
Atrazine	Distilled water	MIP-SPE	Supelco LC-8-DB	Acetonitrile: water (1: 1, v/v)	UV and PDA	Matsui *et al.,* 1997
Atrazine, acetochlor and alachlor	Water	Online SPE (SPE-LC-MS)- styrene-divinylbenzene copolymer	LiChrospher 100 RP-18	Acetonitrile: water gradient	DAD and MS	López-Roldán *et al.,* 2004
Atrazine	Drinking water	No clean-up	Thermo Scientific Hypersil Gold	5 mM ammonium acetate water: methanol gradient	MS/MS	Beck, 2008
Atrazine, terbuthylazine acetochlor and alachlor	Food and fruit	QuEChERS based extraction - PSA and C18 sorbents	Agilent ZORBAX Eclipse Plus C18	10 mM ammonium formate methanol: water gradient	MS/MS	Mastovska *et al.,* 2017
Atrazine	Food crops	QuEChERS- PSA and C18 sorbents	Phenomenex Prodigy ODS-3 150	0.1% formic acid water: acetonitrile gradient	MS/MS	Kwon *et al.,* 2012
Atrazine, terbuthylazine and alachlor	Drinking and lake water	Online SPE (SPE-HPLC-MS/MS)- C18 and biphenyl sorbents	Merck Chromolith SpeedROD RP-18 endacpped	1 mM ammonium acetate methanol: water gradient	MS/MS	Asperger *et al.,* 2002
Atrazine	Water and soil	Online MISPE- LC-DAD	Agilent XDB-C18 column	0.1% formic acid water: methanol gradient	DAD	Zhu *et al.,* 2005

Pesticide(s) of interest analytes	Sample matrix	Clean-up technique	LC column	Mobile phase	Detector	Reference
Atrazine, terbuthylazine and alachlor	Water	No clean-up	MIP- packed column compared to a C18 Phenomenex column	Water: methanol 1:1 (MIP column) water: acetonitrile gradient (C18 column)	DAD	Guzzella *et al.,* 2008
Atrazine and terbuthylazine	Wetland plants	SPE- Florisil and MCX sorbents	Kromasil C8-	0.01 M ammonium acetate water: acetonitrile gradient	DAD	Papadopoulos *et al.,* 2009
Atrazine and terbuthylazine	Ground water	MIP	MIP packed column	Acetonitrile: water gradient	DAD	Ferrer *et al.,* 2000
Acetochlor and alachlor	Ground water	SPE- C18 sorbent	Zorbax SB C-8	0.1% acetic acid water: acetonitrile gradient	MS/MS	Yokley *et al.,* 2002
Atrazine and alachlor	Water	Dummy template MIP	Supelco C-18	Acetonitrile: water (80:20)	DAD	Kueseng *et al.,* 2009

From Table 2.9 it can be seen that the C18 and C8 columns are popular choices for the chromatographic separation of the selected pesticides, whilst an acetonitrile or methanol: water gradient with a small amount of acid or ammonium salt added is the popular choice of mobile phase. UV absorption spectroscopy, often in the form of a diode array detector (DAD), and MS/MS were the most popular detectors used for the analysis of the selected pesticides in these studies.

2.6: Mass spectrometry

From Table 2.8 and 2.9 it is clear MS is commonly used with LC and GC for the analysis of pesticides from a variety of sample matrices. A mass spectrometer (MS) is used to differentiate between background compounds from analytes based on the mass to charge ratio (*m/z*) of their characteristic ions.

There are multiple ways of generating ions for mass spectrometry purposes, and often a specific ion source is selected to be amenable to the chromatographic method that is coupled to the MS. Some common ionization sources include electron ionization (EI), electrospray and chemical ionization. EI is commonly used with GC and was also used for this study.

When identifying a compound using MS, the most important information is the molecular mass of the analyte in question, although this ion may not be present when EI is used. The fragmentation pattern

in the mass spectrum can be very useful, as this can be used to identify unknown compounds, and is necessary for quantitation purposes as both a qualifier and a quantifier ion is necessary for quantitation using single ion recording (SIR) (Patnaik, 2004). SIR can be more generally referred to as single ion monitoring (SIM). This nomenclature differs between manufacturers or software suppliers. The system used for this study, Empower 3 with PerkinElmer SQ8 MS, uses the term single ion recording (SIR).

Atrazine, for example, has the following molecular formula: $C_8H_{14}ClN_5$ and thus has two theoretical molecular ions with *m/z* values of 215 Da (76%) and 217 Da (24%) due to the Cl isotopic abundance. Figure 2.23 shows the electron ionization (EI) mass spectrum obtained from the NIST mass spectrum data centre (NIST, 2014). The five mass fragments with the highest intensity have the following *m/z* ratio: 200; 215; 58; 173 and 202 Da. Figure 2.24 shows these mass fragments on the chemical structure of atrazine and the fragments were calculated as follows:

- *m/z* 215 and 217 relates to M+
- *m/z* 200 and 202 relates to [M-15] due to loss of CH_3 to $C_7H_{11}ClN_5^+$
- *m/z* 173 and 175 relates to [M-42] loss of C_3H_6 to $C_5H_8ClN_5^+$
- *m/z* 58 relates to [M-157] $(CH_3)_2CHNH^+$ (Contains no Cl, 60 *m/z* is not present in the spectrum)

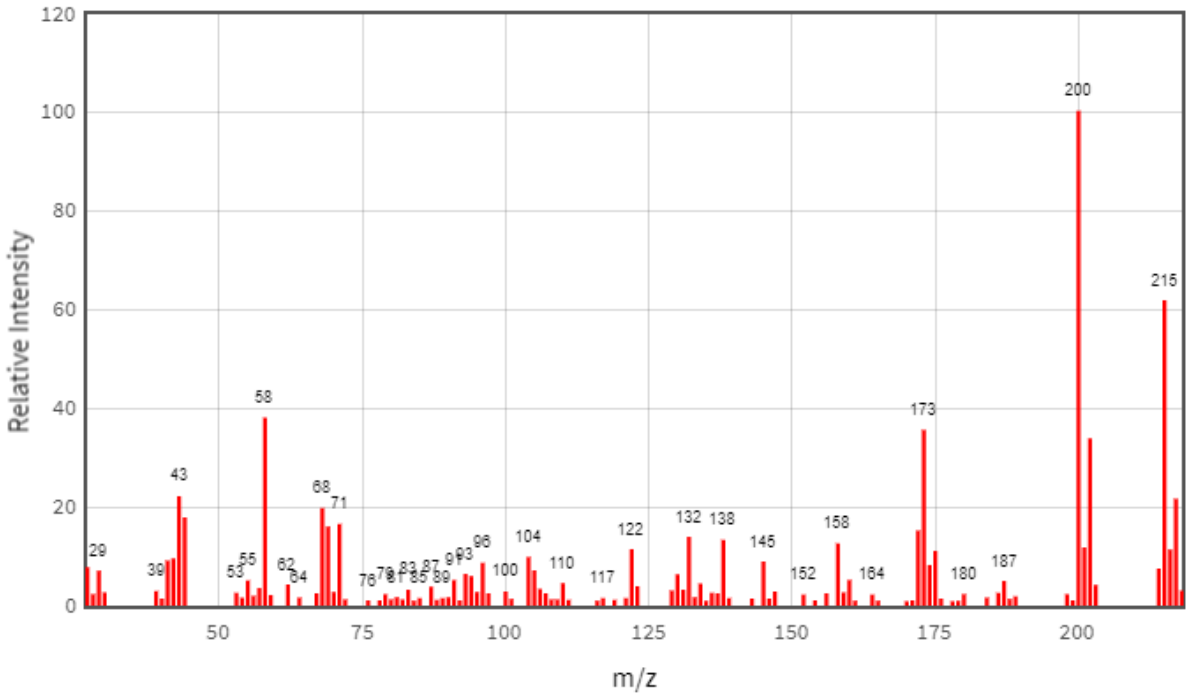


Figure 2.23: Electron ionization mass spectrum for atrazine as reported in the NIST library depicting the mass to charge ratio with relative intensities of the expected fragments (NIST, 2014)

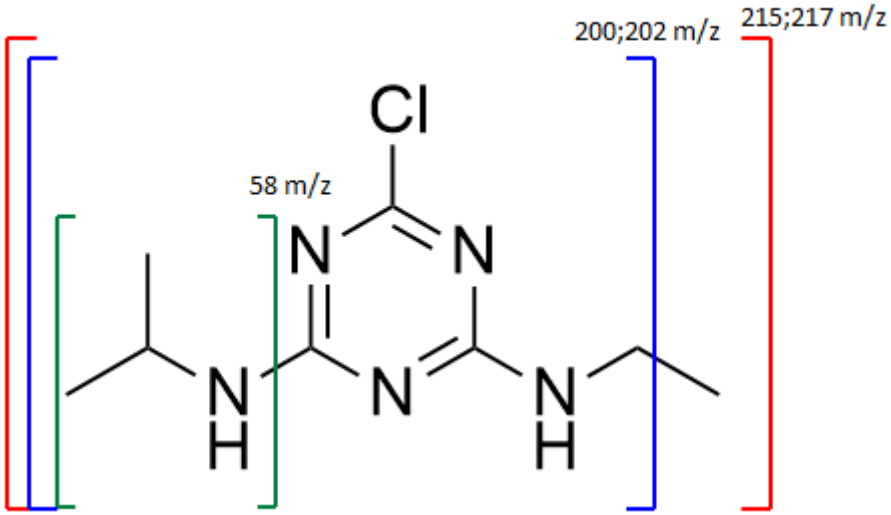


Figure 2.24: Atrazine fragmentation diagram depicting how the major fragments might be formed on the representation of the chemical structure

Refer to the appendix for similar fragmentation diagrams and mass spectra for terbuthylazine, acetochlor and alachlor.

2.7: Molecularly imprinted polymers

2.7.1: Introduction

Molecularly imprinted polymers (MIPs) have been compared to antibodies and have been called ”synthetic antibody mimics” that can be applied to biosensors or SPE (Ferrer *et al.*, 2000; Headley *et al.*, 2010). The selectivity of SPE has been shown to increase when a MIP is employed as a sorbent. It is possible to design MIPs with the purpose of removal or isolation and pre-concentration of target pollutants from aqueous samples (Madikizela & Chimuka, 2016). Advantages of MIPs over immunosorbents include potential reproducibility, and improved stability and load capacity. Disadvantages may include site heterogenicity and slower mass transfer (Ferrer *et al.*, 2000). MIPs have gained an increasing amount of attention, as shown in Figure 2.25, although the idea has been present since the 1930s (Whitcombe *et al.*, 2014).

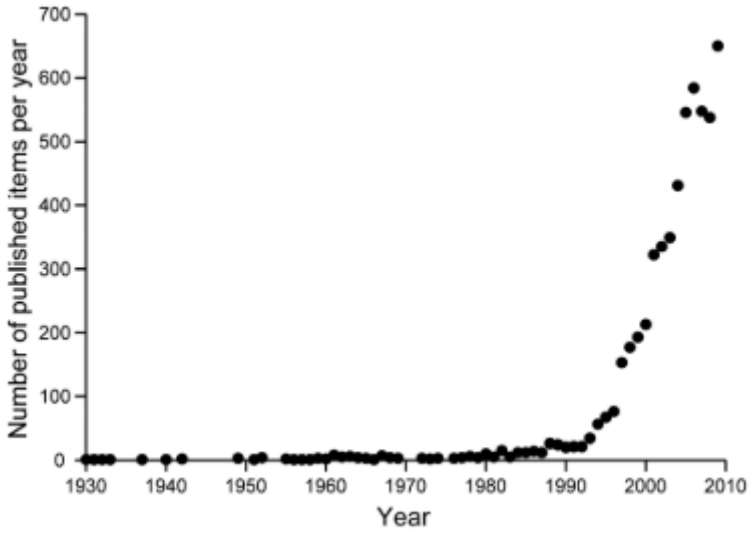


Figure 2.25: Number of publications on MIPs per year from 1931 to 2011 (Whitcombe *et al.*, 2014)

The first use of a MIP as SPE sorbent was described by Börje Sellergren, for the selective determination of the drug pentamidine, used for the treatment of pneumonia related to HIV and AIDS. It was reported that enhanced selectivity was observed for specific enantiomers of the drug, depending on the template used (Sellergren, 1994). The first use of MISPE for the extraction of a triazine pesticide, simazine, from water was described by Matsui *et al*. and was prepared by suspension polymerization. The MISPE method involved the reverse-phase extraction of the hydrophobic agrochemicals from an aqueous solution by orientated hydrogen bonding which demonstrated selectivity towards simazine with 91% recovery, whilst eluting other undesired compounds (Matsui *et al.,* 1997).

Several polymerizable functional monomers have been used in the synthesis of MIPs. The functional monomers prearrange around the template molecule by non-covalent interactions. The polymerization reaction is then initiated and a rigid, highly cross-linked macroporous (pores > 50 nm) polymer is formed that contains cavities that are complementary to the template molecule both in shape and in the arrangement of functional monomers. The template molecule is then removed using a solvent or mixture that the template has an affinity towards. After the template removal, the MIP contains template-shaped cavities that can be used to selectively rebind the template molecules, or related structural analogues, from a sample matrix. Some advantages in the use of MIPs as SPE sorbents include high selectivity, high affinity constants, they are inexpensive and are thermally and chemically stable. There are some disadvantages to consider; MIPs prepared by polymerization need grinding and sieving (size fractionation), which takes time and is a wasteful process. Another major drawback is template bleeding, since the MIP is synthesised using a template molecule, the bleeding thereof from the polymer may influence accuracy and precision of the subsequent analysis. Template bleeding can be countered by effective washing/removal of the template prior to use or by using a

structural analogue for a template molecule (or dummy template), with a similar chemical structure (Headley *et al.*, 2010; Wang *et al.*, 2015).

For triazine pesticides, such as atrazine or terbuthylazine, a structurally similar molecule could be considered as a template molecule, such as triethyl-melamine (Matsui *et al.,* 2000). It was reported that a MISPE with terbuthylazine as template could also be used for the extraction of other triazine pesticides, including atrazine, with a recovery of >80% (Ferrer *et al.*, 2000). The adsorption capacity was, however, not determined or reported. A similar study used butachlor as a MIP template, and both acetochlor and alachlor were then extracted from spiked soil samples using the resulting MIP, with recoveries of 88 and 90% respectively (Wang *et al.*, 2015).

2.7.2: Molecularly imprinted polymer synthesis

A MIP is produced when the polymerization of monomers is initiated in the presence of a template molecule, in this case a pesticide (Figure 2.26). The template molecule is then removed from the polymer, leaving a cavity that is complementary to the template molecule. These cavities have the potential to rebind the target molecule or structural analogues. The polymer thus has affinity for the original template molecule based on covalent bonds, non-covalent bonds, electrostatic interactions, hydrophobic or van der Waals interactions, ligand exchange or co-ordination with a metal centre, as shown in Figure 2.26 (Whitcombe *et al.,* 2014).

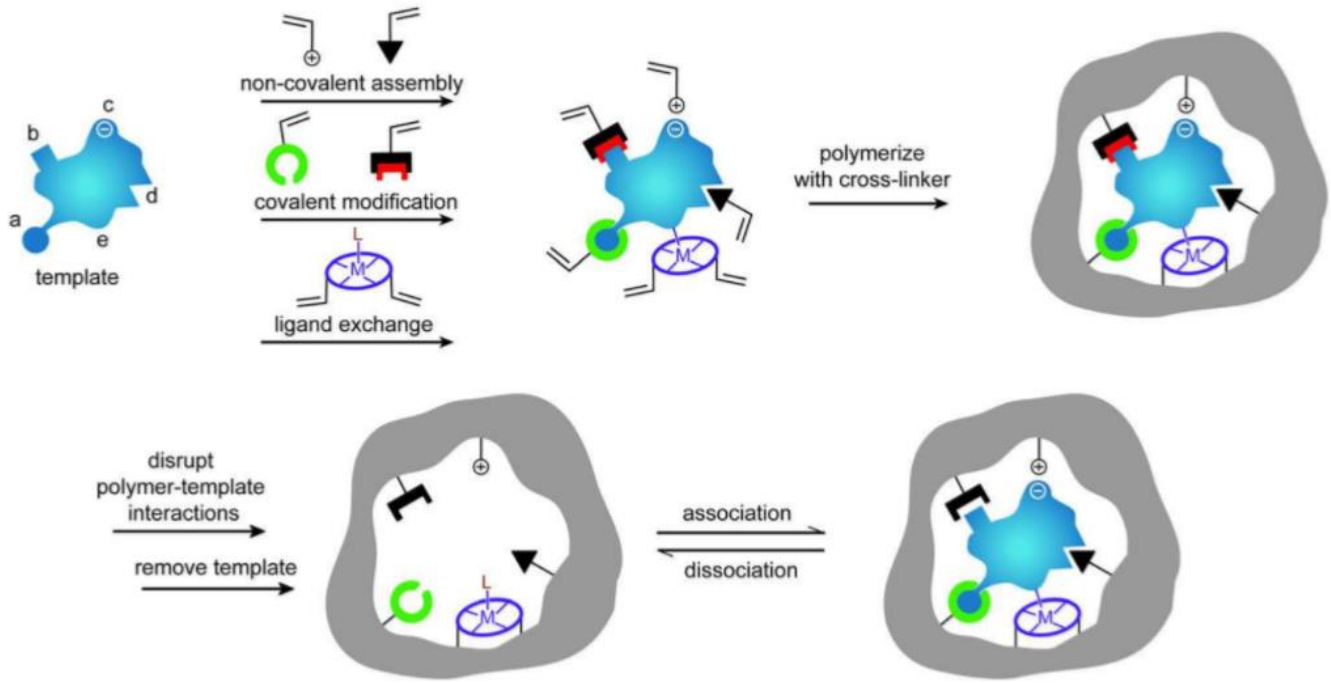


Figure 2.26: Schematic representing the interaction of the MIP with the template molecule (Whitcombe *et al.,* 2014)

Several MIPs for pesticide extraction applications have been reported in the literature. For example, four organophosphate pesticides were extracted from soil and water samples using self-manufactured molecularly imprinted solid phase extraction cartridges (Zhu *et al*., 2005). The MIP-SPE technique performed well in terms of selectivity and limit of quantitation when compared to a classic ENVI-18 SPE technique (Zhu *et al*., 2005). Methacrylic acid (MAA) and ethylene glycoldimethacrylate (EGDMA) were cleaned to remove the inhibitor prior to polymerization reactions to form the MIP with the organophosphate pesticides as template. Azobisisobutyronitrile (AIBN) was used as an initiator for the polymerization reaction. For polymer preparation, 1 mmol of template (pesticide) and 4 mmol of MAA were dissolved in dichloromethane. The EDGMA cross-linker (20 mmol) and the AIBN initiator (0.23 mmol) were added to the mixture which was purged with nitrogen. The tube was sealed under vacuum and placed in a shaker bath at 58 °C for 24 h. As a reference, a non-imprinted polymer was simultaneously prepared in the same way but without the addition of the template (Zhu *et al*., 2005).

The bulk polymer was crushed, ground and wet sieved with acetone to collect a particle size fraction of 40–60 µm. The organophosphate pesticide template was washed out of the sieved MIP fraction with 10% acetic acid and methanol solution. The MIP fraction was then washed with methanol to remove residual acetic acid and subsequently dried. PTFE frits were used inside a PTFE SPE cartridge above and below the MIP sorbent bed to pack the MISPE cartridge (Zhu *et al*., 2005).

A very similar MIP synthesis method was described by Kueseng et al. (2009), where instead of DCM, toluene was used as porogen. MIP particles in the range 38—106 µm were collected and placed in a Soxhlet extraction apparatus and washed with methanol for 48 h and dried in a desiccator for 12 h (Kueseng *et al.*, 2009).

Although the method described by Chen *et al.* (2014) employs a different approach, using ultraviolet (UV) and far-infrared (FIR) in the synthesis, as illustrated in Figure 2.27, the procedure is still highly applicable, as the target analyte was atrazine. Atrazine MIPs were comparatively synthesised using the identical polymer formulation by FIR radiation and UV-induced polymerization using methacrylic acid (MAA) as the functional monomer, ethylene glycol dimethacrylate (EGDMA) as the crosslinker, dichloromethane as the porogen, and 2,2'-azobisisobutyronitrile (AIBN) or Irgacure 1800 as the FIR-radiation initiator or UV- initiator, respectively. Irgacure 1800 is a trade name for a solid photo initiator for radical polymerisation of unsaturated resins after UV light exposure. Atrazine (1 mmol) and methacrylic acid (4 mmol) were left in contact for 30 min for pre-arrangement. Subsequently, EDMA (20 mmol), AIBN (0.24 mmol) and 5 mL of chloroform (used as porogen) were added. The mixture was purged with N_2 and the bottle was sealed under this atmosphere. These samples were then placed in the FIR heater with rotation for even irradiation at a controlled temperature of 65 °C for 18 h to carry

out the polymerization process. In comparison, 0.04 g photo-initiator (Irgacure 1800) was added instead of AIBN to the above solutions using the identical polymer formulation, but the sealed tubes were irradiated with a UV lamp in an ice bath for 6 h. After polymerization, the polymers were ground and sieved. Soxhlet extraction was performed to remove the template molecule, a two-step procedure repeated four times: acetic acid and methanol (1:9, v/v) was firstly used for 12 h, followed by methanol for 6 h. Particles with size between 47 and 74 µm were collected and then repeatedly suspended in acetone to remove the small particles (Chen *et al.*, 2014).It is unclear why the sedimentation step was required after the Soxhlet extraction. Perhaps the sieving was not effective or the Soxhlet formed smaller particles again after they had been removed.

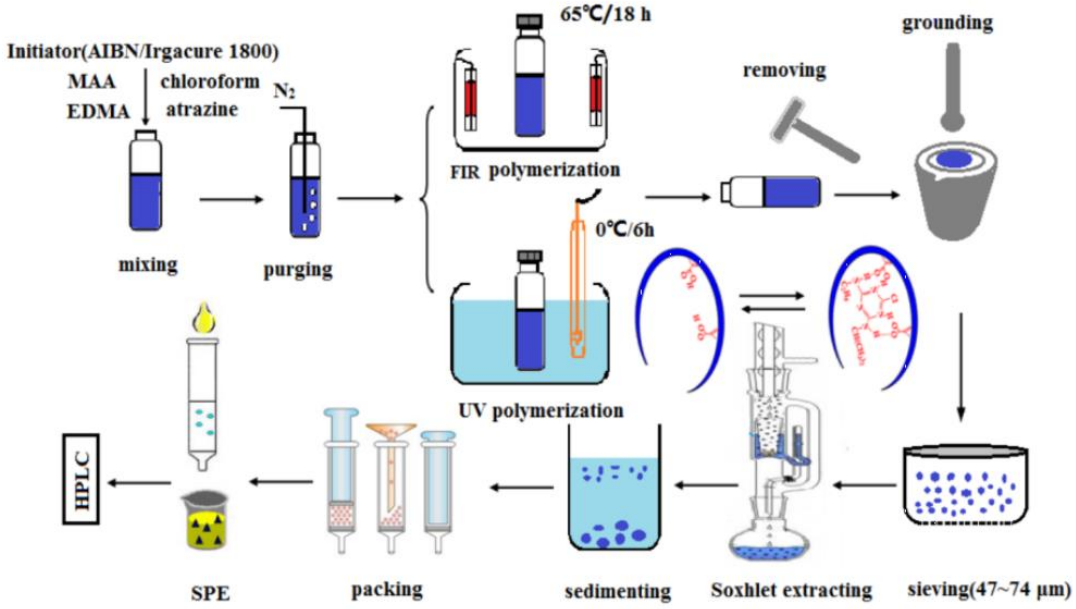


Figure 2.27: Schematic representation showing the preparation of atrazine imprinted polymers for SPE (Chen *et al.*, 2014)

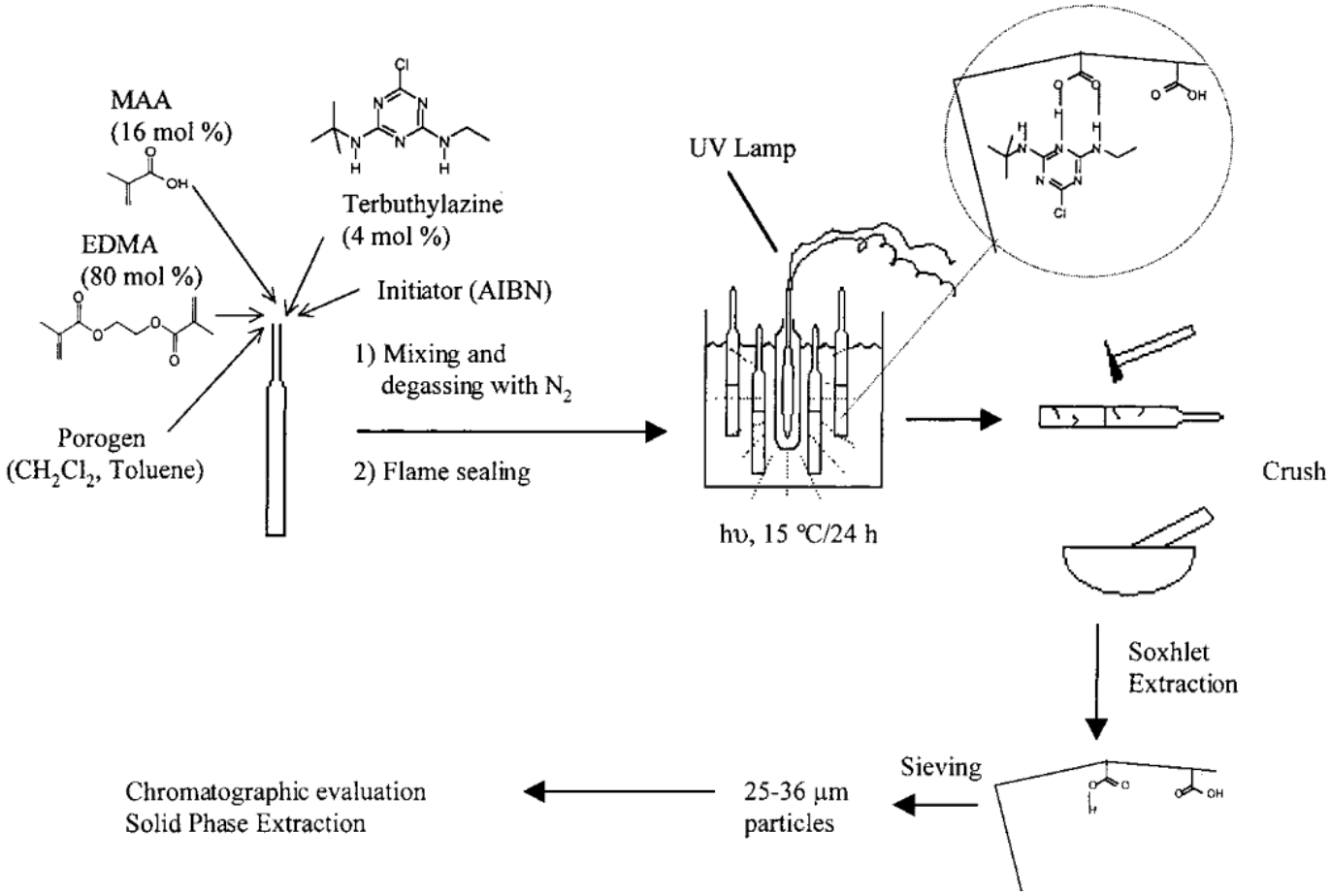


Figure 2.28: A flow diagram representing the synthesis and preparation procedure for a terbuthylazine MIP (Ferrer et al., 2000)

Figure 2.28 shows another schematic for the synthesis of a terbuthylazine MIP, which uses UV as an activator for the AIBN initiator, and dichloromethane and toluene were used for the separate synthesis of two MIPs respectively. The template was removed with three Soxhlet cycles, using methanol: water (50:50), methanol and methanol: acetic acid (90:10), respectively (Ferrer *et al.*, 2000).

The following procedure was described by Guzzella, Pozzoni and Bagguani (2008) and includes commonly used triazanes (atrazine and terbuthylazine) and their widespread metabolites (desethyl-atrazine and desethyl-terbuthylazine) as templates for MIP synthesis. The MIP was produced by bulk polymerisation using MAA as functional monomer, EDGMA as cross-linker, propazine as template and toluene as porogen solvent. Three different washing methods were tested to remove the template molecules from the MIPs. The first involved eluting cartridges with methanol: acetic acid (9:1, v/v). In the second extraction method a microwave was utilized to extract the polymers at 100 °C for 20 to 40 min in methanol: acetic acid (1:1, v/v). The last extraction method worked the best and involved an automated Soxhlet apparatus for 20 extraction cycles and 40 min washing steps using methanol: acetic acid (9:1, v/v) (Guzzella *et al.,* 2008).

Atrazine MIP nanoparticles were reportedly synthesised by a precipitation polymerization process using various monomers (Gkementzoglou *et al.,* 2013). EDGMA was used as a cross linker and AIBN as an initiator, with acetonitrile and toluene as porogens. Acrylamide, MAA, itaconic acid and 2-(trifluoromethyl) acrylic acid (TF-MAA) were used as monomers. Different combinations of monomers and porogens were utilized and the resulting nanoparticle MIPs were characterized and tested for template retention and rebinding capacity. Subsequently, the template molecule was extracted from the polymeric particles by means of a series of successive washing cycles with a methanol: acetic acid solution (9:1, v/v). The excess acetic acid was then removed with methanol. Non-imprinted polymers (NIP) were also prepared under the same polymerization conditions in the absence of the template molecule. Figure 2.29 shows SEM images of atrazine MIP nanoparticles that were obtained utilising different monomers and porogens (Gkementzoglou *et al.*, 2013).

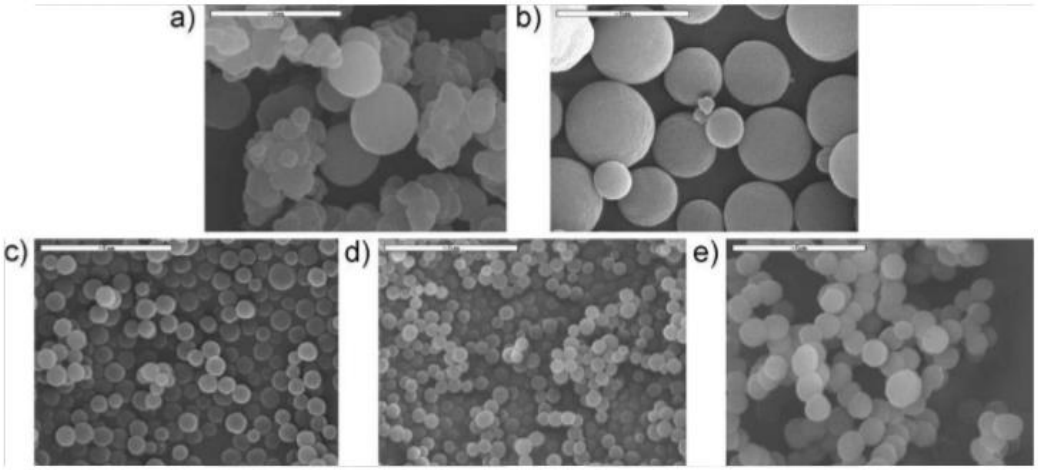


Figure 2.29: SEM images of atrazine MIP nanoparticles obtained from the different monomer combinations. a) is TF-MAA monomer, b) acrylamide monomer, c) itaconoc acid monomer, d) MMA monomer with acetonitrile as porogen and e) MMA with toluene as porogen
(Gkementzoglou *et al.*, 2013)

With reference to Figure 2.29, when the TF-MAA monomer was used (a) the product appeared to contain irregularly shaped MIPs mixed with spherical ones. The acrylamide monomer formed larger spherical MIPs that were much more uniform in shape (b). The remaining MIPs (c to e) all appeared to be much finer particles with a larger surface to volume ratio than (a) and (b).

It has been reported that computational techniques have been used to identify the best suited functional groups for MIP synthesis, as in the following example and illustrated in Figure 2.30.

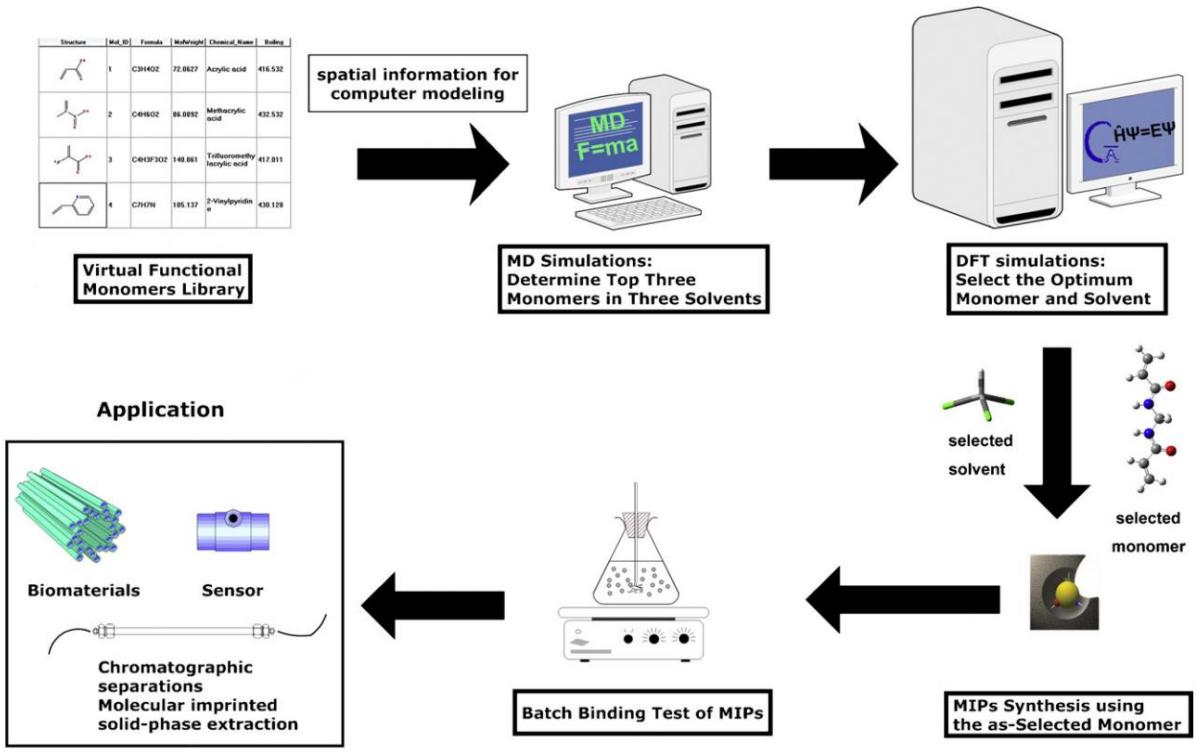


Figure 2.30: Flow diagram of the computational flowchart for MIP synthesis and application (Dong *et al.*, 2009)

The template, acetochlor, was dissolved in the porogen (chloroform), and mixed with N,N-methylene bisacrylamide monomer. The cross-linker divinylbenzene and free radical initiator AIBN were added into the solution, which was purged with nitrogen to remove oxygen, sealed and incubated at 65 °C for 24 h in a water bath. The resultant bulk polymer (MBAAM-MIP) was washed with methanol-acetic acid (9:1, v/v) repeatedly until acetochlor could not be detected in the filtrate. The polymer complex was dried under vacuum. The synthesised bulk polymer was ground with an agate mechanical mortar. Another imprinted polymer (AAM-MIP) was synthesised using the same approach stated above with acrylamide as monomer and acetonitrile as porogen (Dong *et al.*, 2009).

Alachlor and acetochlor, along with 2 other chloroacetamide herbicides, were extracted from soil simultaneously using a butachlor dummy template MIP. MAA and EDGMA were used as functional monomer and crosslinker respectively. Acetonitrile was employed as porogen with AIBN as activator. Nitrogen was used to purge the mixture prior to polymerization at 60°C in a water bath for 24 h. Methanol: acetic acid (9: 1, v/v) was used to remove the pesticide template (Wang *et al.*, 2015).

Table 2.10 contains examples of literature MIP synthesis for the target pesticides highlighting the differences in terms of monomers, cross linkers initiators and porogens used. The template removal technique was also highlighted.

Table 2.10: Summary of MIP synthesis procedures for the target pesticides as reported in the literature

Pesticide	Monomer	Linker	Initiator	Porogen	Template removal	Reference
Atrazine	MAA	EDGMA	AIBN	Dichloromethane	MeOH/acetic acid (9:1 v/v) for 6 h + MeOH for 12 h Soxhlet Repeated 4 times	Chen *et al.,* 2014
Atrazine	MAA	EDGMA	AIBN	Toluene	MeOH for 48 h Soxhlet	Kueseng *et al.,* 2009
Atrazine	MAA	EDGMA	AIBN	Toluene	MeOH/acetic acid (9:1 v/v) for 20 cycles	Guzzella *et al.,* 2008
Atrazine	MMA/ Itaconic acid /Acrylamide/ TFMAA	EDGMA	AIBN	Acetonitrile/ Toluene	MeOH/acetic acid (9:1 v/v) *The number of repeats not stated*	Gkementzoglou *et al.,* 2013
Atrazine	MAA	EDGMA	AIBN	Toluene	MeOH/acetic acid (7:1 v/v) Soxhlet *Time not stated*	Geng *et al.,* 2015
Terbuthylazine	MAA	EDGMA	AIBN	Toluene	MeOH/acetic acid (9:1 v/v) for 20 cycles	Guzzella *et al.,* 2008
Terbuthylazine	MAA	EDGMA	AIBN	Toluene/ Dichloromethane	MeOH/water (50/50), MeOH, MeOH/acetic acid (90/10)	Ferrer *et al.,* 2000
Acetochlor	MBAAM/ Acrylamide	DVB	AIBN	Chloroform/ Acetonitrile	100 mL MeOH/acetic acid (9:1 v/v) until acetochlor could not be detected in the filtrate	Dong *et al.,* 2009
Acetochlor	MAA	EDGMA	AIBN	Acetonitrile	MeOH/acetic acid (9:1 v/v) *The number of repeats not stated*	Wang *et al.,* 2015

Pesticide	Monomer	Linker	Initiator	Porogen	Template removal	Reference
Alachlor	MAA	EDGMA	AIBN	Acetonitrile	MeOH/acetic acid (9:1 v/v) *The number of repeats not stated*	Wang *et al.,* 2015
Alachlor	o-Phenylene diamine	None	Electro-polymer-ization	Acetate buffer solution	Electrode submersed in a stirring ethanol-water (2:1, v/v) followed by MeOH/acetic acid (9:1 v/v) submerged stirring for 20 min	Elshafey & Radi, 2018

A molecularly imprinted polymer for alachlor has been used in conjunction with an electrochemical impedance sensor. The monomer used was o-phenylenediamine, without a crosslinker, and polymerization was achieved utilizing electro-polymerization over a polished glassy carbon electrode. The synthesised electrode could detect when alachlor was bonded in the MIP cavities (Elshafey & Radi, 2018).

2.7.2.1: Polymerization inhibitor

The polymerization reagents, such as 0.2 M AIBN in toluene, typically contain 30 ppm 4-methoxyphenol, denoted as MEHQ (Figure 2.31), polymerization inhibitor. Both MAA and EDGMA also usually contain 250 and 100 ppm MEHQ, respectively. The MEHQ is added to enhance shipping stability at an ambient temperature of the polymerization reagents as well as to enhance the shelf life and expiry date of such items (Armarego & Chai, 2008). Some studies suggest recrystallization of AIBN from methanol in order to remove this inhibitor (Wang *et al.*, 2015) and others suggest standard clean-up methods for MAA and EDGMA to remove the initiator (Matsui *et al.,* 2000). Another literature source also referred to a cleaning procedure for MAA and EDGMA (Zhu *et al*., 2005).

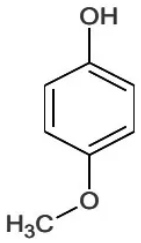


Figure 2.31: 4-Methoxyphenol, also referred to as MEHQ or mequinol, commonly used as a polymerization inhibitor

The MEHQ polymerization inhibitor can be removed from MAA by extraction with a 10% NaOH solution, whereby the inhibitor is extracted into the lower aqueous layer. The MAA in the top layer can then be dried with a suitable dehydration agent, like sodium sulphate (Armarego & Chai, 2008). For EDGMA an alumina column is suggested, as this will separate EDGMA from MEHQ. The solvent is then evaporated under vacuum, and care must be taken not to heat the polymerization monomers without the polymerization inhibitor (Armarego & Chai, 2008).

2.7.2.2: Polymerization reaction mechanism

The polymerization reaction mechanism described in this section is for the preparation of ethylene glycol dimethacrylate (EGDMA)- methacrylic acid (MAA) copolymers for use in MIP synthesis, using 2,2' -azobisisobutyronitrile (AIBN) as polymerization activator. MAA (Figure 2.32) and EDGMA (Figure 2.33) are referred to as the functional monomer and crosslinker respectively.

AIBN (Figure 2.34) functions as the polymerization initiator during the MIP synthesis. The AIBN molecule self-decomposes, under heat or UV exposure, to form a molecule of N_2 and two 2-cyanoprop-2-yl radicals, refer to Figure 2.35. These free radicals initiate the polymerization reaction, known as free-radical polymerization (Ramelow & Pingili, 2010). The initiation reaction can be achieved at high (60 °C) or low temperature (0 °C). There should not be oxygen present in the reaction, otherwise the oxygen free radicals can capture free radicals and polymerization will be blocked (Niu *et al.,* 2016). The polymerization reaction can also be referred to as precipitation polymerization, as all the individual monomers are fully soluble in toluene but the polymer precipitates out of solution (Gkementzoglou *et al.*, 2013).

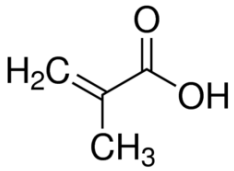


Figure 2.32: Chemical structure of methacrylic acid (MAA)

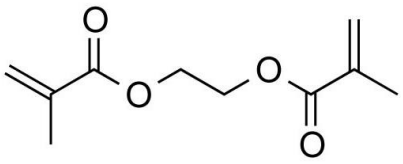


Figure 2.33: Molecular structure of ethylene glycol dimethacrylate (EGDMA)

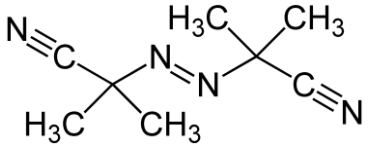


Figure 2.34: Molecular structure of 2,2'-azobis(2-methylpropionitrile) (AIBN)

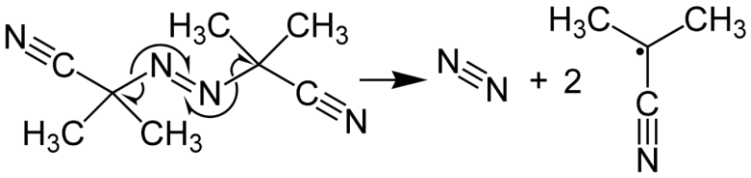


Figure 2.35: The self-decomposition of AIBN to form nitrogen gas and two free radicals (Ramelow & Pingili, 2010)

The reaction mechanisms described in Figure 2.36 to 2.41 are very similar to the ones described in literature, except methyl methacrylate was used (Ramelow & Pingili, 2010), instead of methacrylic acid (MAA). The radical initiator reacts with an unsaturated hydrocarbon thus the methyl-ether functional group does not partake in the reaction mechanism and the reaction with MAA is expected to be similar. Figure 2.36 and 2.37 illustrate how the initiator radical, formed in Figure 2.35, activates EDGMA and MAA respectively to initiate the polymerization reaction. The continued polymerization reactions are depicted in Figure 2.38 and Figure 2.39 to form I-EDGMA-EDGMA$^{\bullet}$ and I-MAA-MAA$^{\bullet}$ polymer chains.

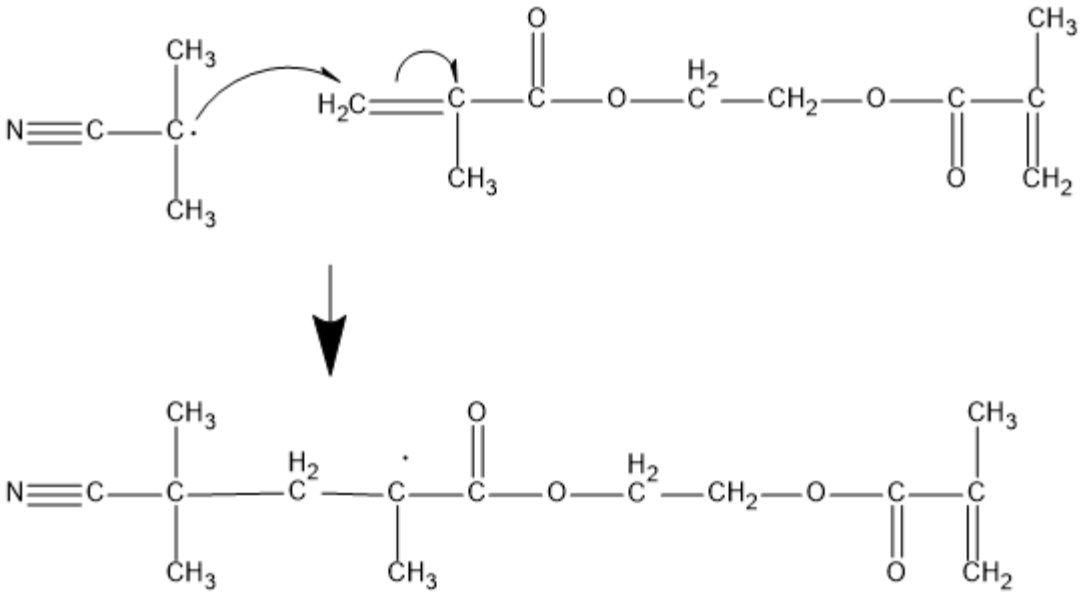


Figure 2.36: The initiation of EDGMA by the AIBN radical to from I-EDGMA•

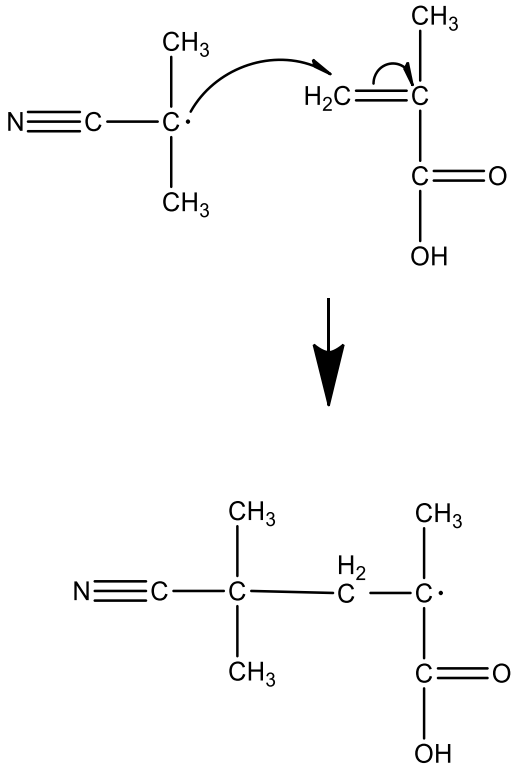


Figure 2.37: The initiation of MAA by the AIBN radical to from I-MAA•

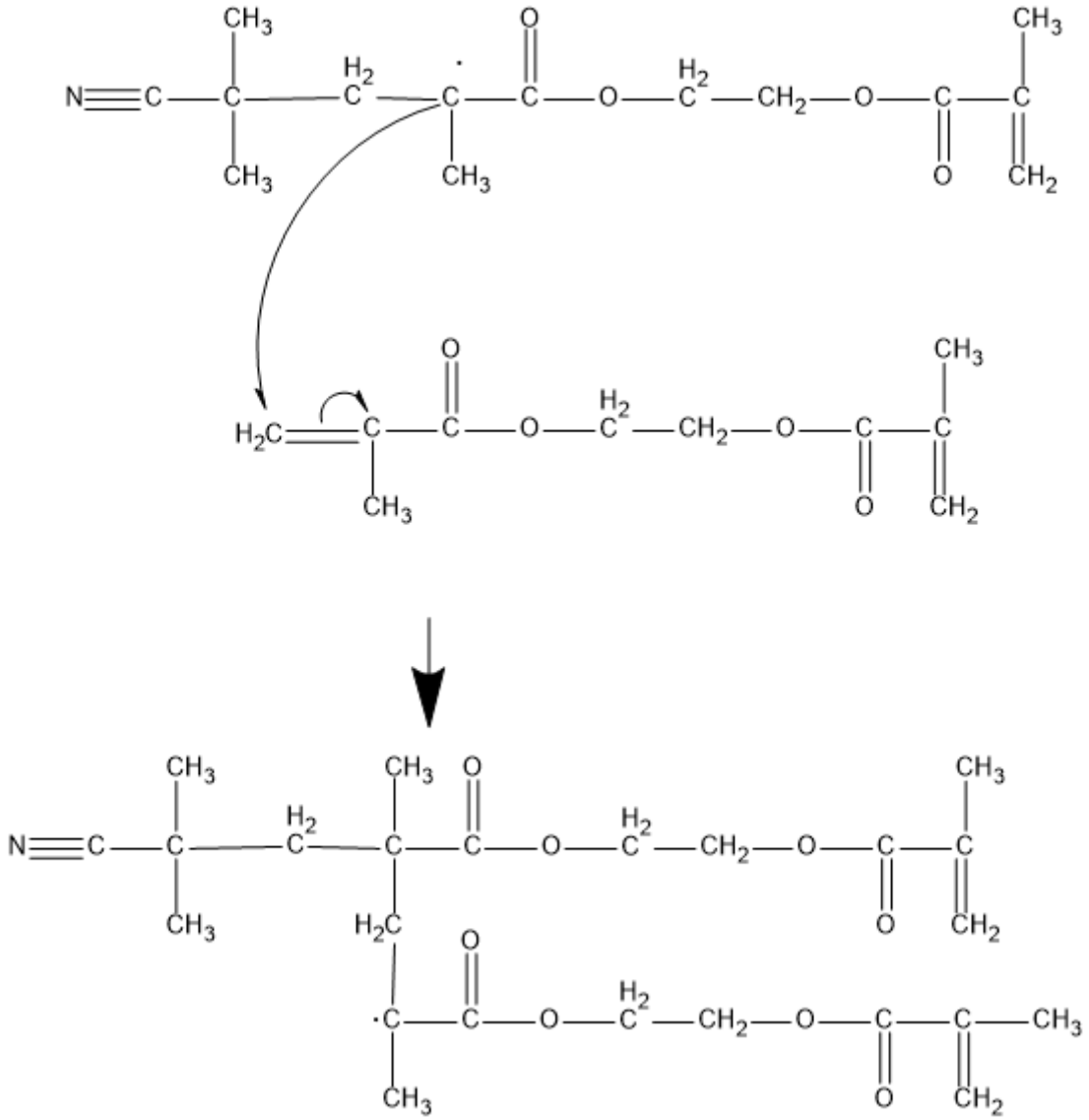


Figure 2.38: The continued polymerization of the I-EDGMA• radical with another EDGMA molecule to form I-EDGMA-EDGMA•

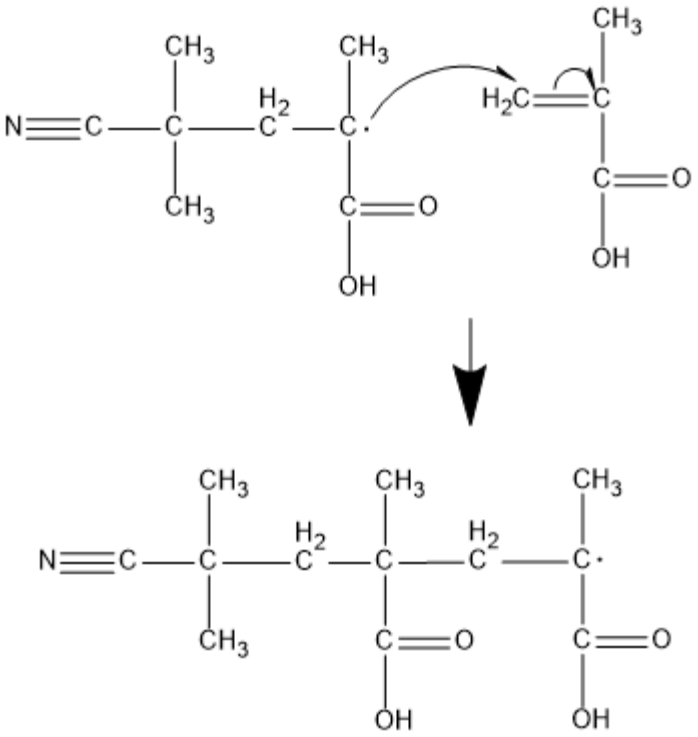


Figure 2.39: The continued polymerization of the I-MAA• radical with another MAA molecule to form I-MAA-MAA•

Mixed polymer formations are also possible, where I-MAA-EDGMA• and I-EDGMA-MAA• polymer chains are formed, as depicted in Figure 2.40 and Figure 2.41.

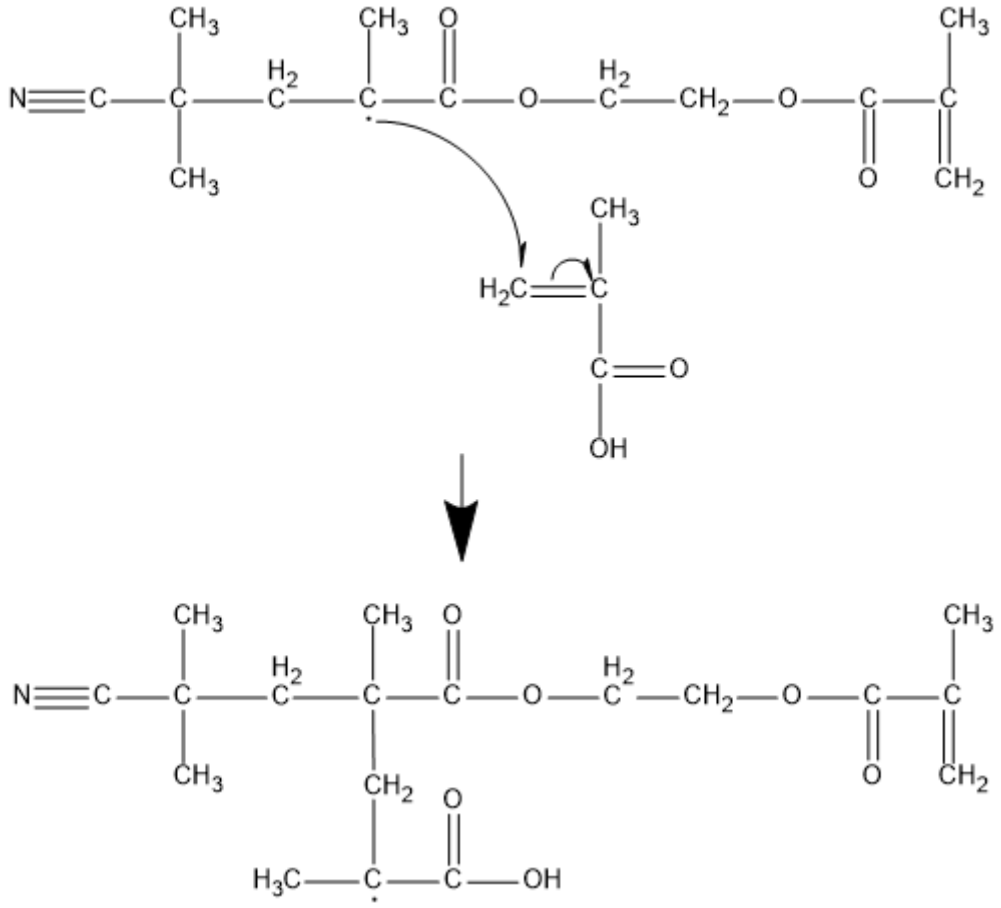


Figure 2.40: The continued polymerization of the I-EDGMA• radical with a MAA molecule to form I-EDGMA-MAA•

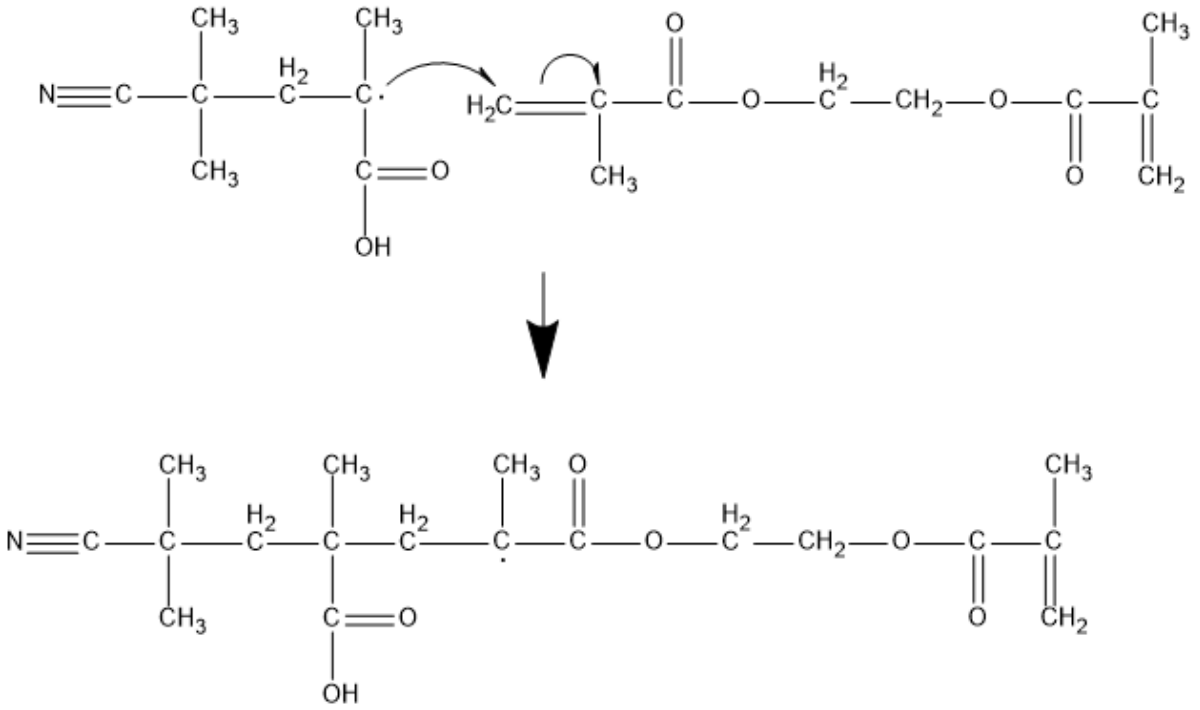


Figure 2.41: The continued polymerization of the I-MAA• radical with a EDGMA molecule to form I-MAA-EDGMA•

A summary of all the possible reactions following the above reaction mechanisms are listed below:

I-EDGMA-EDGMA• + EDGMA → R-EDGMA•

I-MAA-MAA• + EDGMA → R-EDGMA•

I-MAA-EDGMA• + MAA → R-MAA•

I-EDGMA-MAA• + MAA → R-MAA•

Where "R" represents the previously formed polymer. R-MAA• and R-EDGMA• represent growing polymer chains ending in MAA and EDGMA respectively. Both R-MAA• and R-EDGMA• can react again with another MAA or EDGMA molecule, thus growing the EDGMA-MAA copolymer.

EDGMA functions as a cross linker, and as seen in Figure 2.33, the molecule is symmetrical. This means that an activated EDGMA molecule already present in an EDGMA-MAA copolymer chain, has another open reaction site where it can be activated again by an AIBN initiator molecule or another growing polymer chain can once again attach to it. This allows for cross linking of polymers and thus the eventual formation of a lattice, which is how the MIP pores are formed and how they are kept rigid after the template molecule is washed out.

The schematic in Figure 2.42 represents a possible EDGMA-MAA copolymer, with the intention of showing how EDGMA functions as a cross-liker in the copolymer.

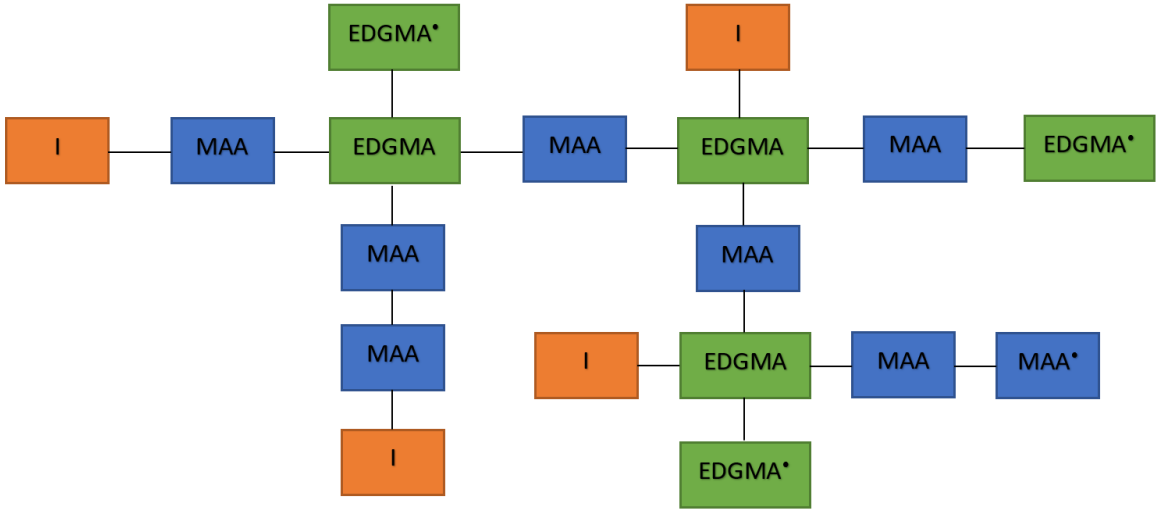


Figure 2.42: Schematic of a hypothetical EDGMA-MAA copolymer intended to show how EDGMA might function as a cross-linker allowing the eventual formation of a lattice and rigid cavities

2.7.2.3: Self-orientation of functional monomers in the MIP cavities

Due to the presence of the carboxylic group on the MAA functional monomer, MAA self-orientates to form hydrogen bonds with the template molecule, with the orientation depending on the functional groups present on the template. For example, hydrogen bonding is possible between the MAA molecule and amino or hydroxyl groups. MAA based MIPs possess strong binding ability and specific capacity (Niu *et al.,* 2016).

Two hydrogen bonds are expected to be formed between atrazine and MAA. This is considered a key interaction necessary for orientation of MAA in the formation of binding sites. The carboxylic group of MAA functions as both a hydrogen bond acceptor and donor, interacting with a hydrogen atom of the amino group and a nitrogen atom of the triazine molecule as depicted in Figure 2.43.

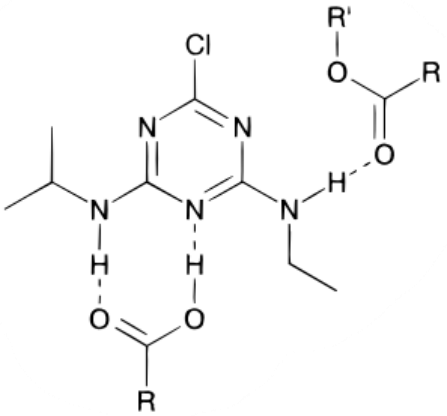


Figure 2.43: Schematic representation of possible hydrogen bonds formed with atrazine during MIP synthesis containing methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDGMA) (Matsui *et al.,* 2000)

It should be noted that no direct evidence has been shown for this self-orientation, although it is reasonable to assume that two or more hydrogen bonds are formed with MAA and perhaps with EDMA molecules during the imprinting process because of the selective binding properties of the MIP (Matsui *et al.,* 2000). Another study confirmed this self-orientation and double interaction between MAA monomer and triazine template with computational modelling (Figure 2.44). The template however was propazine, which is very similar in structure to atrazine and terbuthylazine.

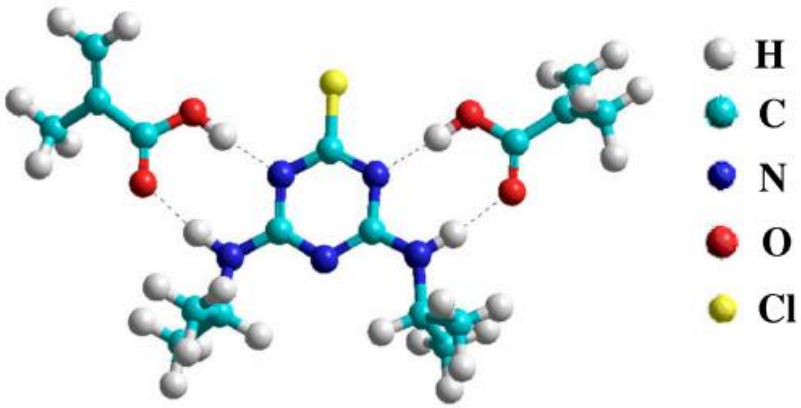


Figure 2.44: Computational hydrogen bond interactions between MAA and propazine demonstrating self-orientation of MAA due to two interactions with the triazine template (Geng *et al.*, 2015)

Based on the hydrogen bonding of atrazine as described in literature (Matsui *et al.,* 2000), possible hydrogen bonds for terbuthylazine, acetochlor and alachlor are shown in Figure 2.45, Figure 2.46 and Figure 2.47 respectively.

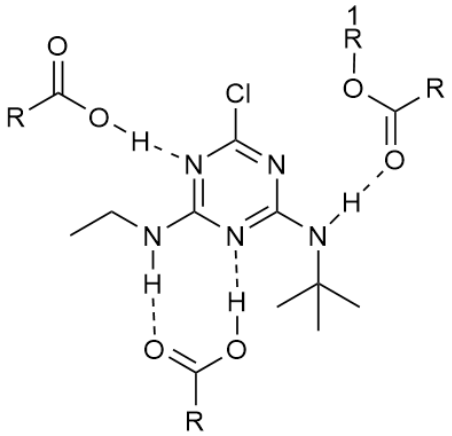


Figure 2.45: Schematic representation of possible hydrogen bonds formed with terbuthylazine during MIP synthesis containing methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDGMA)

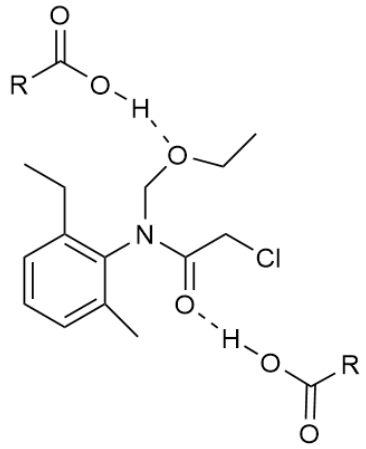


Figure 2.46: Schematic representation of possible hydrogen bonds formed with acetochlor during MIP synthesis containing methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDGMA)

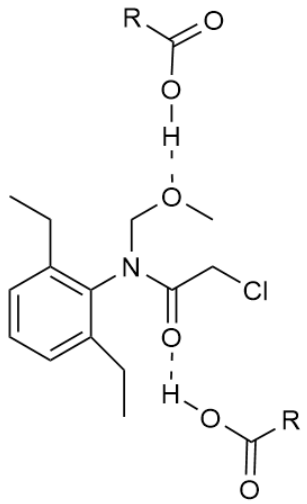


Figure 2.47: Schematic representation of possible hydrogen bonds formed with alachlor during MIP synthesis containing methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDGMA)

Another literature example (Figure 2.48) suggests that the tertiary amine can also undergo hydrogen bonding, suggesting three possible hydrogen bonding sites for acetochlor and alachlor.

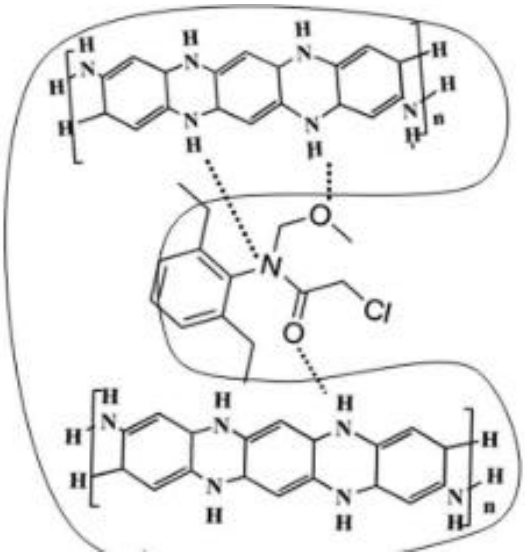


Figure 2.48: Example of hydrogen bonding and monomer orientation of a o-phenylenediamine MIP with an alachlor template (Elshafey & Radi, 2018)

From the possible hydrogen bonding depicted in Figure 2.43 to 2.48 between the MAA and EDGMA monomers functional groups and the template molecules, it is clear that there are more hydrogen bonding sites for atrazine and terbuthylazine, with five amines, than for acetochlor and alachlor with a maximum of three hydrogen bonding sites with an amide and ether functional groups. This would suggest the triazine pesticides might have a stronger affinity for their complementary cavities than that of the chloroacetamide pesticides. It would also be reasonable to consider atrazine and terbuthylazine to be more prone to forming a random hydrogen bond with the NIP.

Chapter 3 : Experimental methods

Materials and equipment used

Methacrylic acid (MAA) containing 250 ppm 4-methoxyphenol (MEHQ) inhibitor, ethylene glycol dimethacrylate (EDGMA), 98%, and 0.2 M 2,2'-azobis(2-methylpropionitrile) (AIBN) in toluene were all purchased from Sigma-Aldrich (Germany). Powder or "neat" standards used as templates in MIP synthesis, namely atrazine, terbuthylazine, acetochlor and alachlor, were also purchased from Sigma-Aldrich from the Pestanal® range. HPLC grade toluene, methanol, and glacial acetic acid were also purchased from Sigma-Aldrich (Germany). In addition to the neat standards previously mentioned, 100 µg/mL atrazine, terbuthylazine, acetochlor and alachlor solutions were purchased from Separations (South Africa) from the Accustandard® (Connecticut, USA) range supplied in 1 mL methanol. Refer to the appendix for the certificates of analysis for each of the aforementioned standards. Silanization solution was prepared by diluting dimethylchlorosilane (DMDCS) 10% in HPLC grade toluene, both purchased from Sigma-Aldrich (Germany). Sodium chloride (99.8%), caffeine (99.9%) and HPLC grade acetonitrile (99.8%) were purchased from Sigma-Aldrich (Germany). Ultra-pure helium (> 99.99%) was purchased from Airproducts (South Africa) and used as GC carrier gas. Water obtained from a Millipore Milli-Q Synthesis A10 water purification system was used for preparation of aqueous pesticide solutions and wash fractions.

A Visiprep™ 24 SPE vacuum manifold from Supelco (supplied by Separations, South Africa) with an Edwards 1.5 oil vacuum pump was used for the packing of the SPE cartridges and later for SPE purposes as well. Two stackable 200 mm stainless steel sieves from Labotec (Pty) Ltd (South Africa) with apertures of 53 and 25 µm respectively were employed for size fractionation of the synthesised polymers prior to packing. Strata C18-E SPE cartridges from Phenomenex (supplied by Separations, South Africa) with a bed-volume of 100 mg in a 3 mL syringe were used as a reference to the MISPE.

A vacuum sample concentrator from CHRIST (RVC-218-CDPlus) equipped with a Julabo F25-EC chiller and a Vacuubrand chemistry diaphragm pump MZ 2C NT (system supplied by Separations) was used for the concentration of small samples < 2 mL. A Mettler Toledo AB104-S/FACT balance (Sigma-Aldrich) was used for weighing purposes. An ultrasonic bath, (ScienTech, Labotec) was used to homogenise or agitate samples or solution and was also found to be effective for template removal. A Thermo Scientific MaxiMix™ II Vortex Mixer was used to mix solutions or to homogenise samples before analysis. MIPs and samples were centrifuged using a Hettich zentrifugen, Rotofix 32 A (Labotec (Pty) Ltd, South Africa). A decommissioned Agilent GC was used as a drying oven. An Isotemp 145 D heating block (Fisher Scientific, South Africa) was also used.

Vials, including the 1 mL GC vials, and larger 15 mL crimp cap headspace vials were purchased from Macherey Nagel (supplied by Separations). For transferring of liquids, single channel Brand pipettes (100 to 1000 µL, 20 to 200 µL and 2 to 20 µL) were used from the Transferpette S digital series (Sigma-Aldrich, Germany). For larger volumes, a Brand Handy Step electronic pipette (used with 25 mL tips) was employed. The pipettes were calibrated at least annually at LABCAL Solutions, which is SANAS accredited.

Cannabis samples were spiked with CRMs to simulate background matrix during extraction. A Kambrook 500 W power drive blender (bought at Game) was used to homogenise cannabis buds into a fine powder. LaPhaPack 5 mL Luer-Loc disposable syringes (supplied by Separations) equipped with Sartorius 0.45 µm PTFE syringe filters (Sartorius South Africa) were used to filter the cannabis sample extracts before SPE to prevent frit blocking.

3.1: MIP synthesis

3.1.1: NIP synthesis procedure

Before a MIP synthesis was performed, a non-imprinted polymer (NIP) reaction was conducted to first optimize the polymerization reaction and to determine if polymerization occurred without the removal of the MEHQ polymerization inhibitor in the monomers and AIBN activator.

The synthesis procedure was based on literature (Kueseng *et al.*, 2009), although the scale of the reaction was decreased to one tenth of that reported, but the same molar ratios were used for the monomers.

Three repeats of the NIP synthesis were performed increasing the amount of AIBN initiator each time (Table 3.1) while keeping the MAA and EDGMA amounts constant. The toluene volume was adjusted to maintain a constant final volume of 700 µL for all three reactions.

Table 3.1: Summary of the reagents used during the three NIP synthesis reactions

Reagents		Amount added (mmol)	Molar mass (g/mol)	Mass (mg)
MAA (methacrylic acid)		0.4	86.06	34.4
EDGMA (ethylene glycol dimethacrylate)		0.4	198.22	79.2
Reagents in Solution		**Amount added (mmol)**	**Concentration of stock solution**	**Volume (µL)**
1	AIBN	0.03	0.2M in toluene	150
	Toluene	-	-	550
2	AIBN	0.06	0.2M in toluene	300
	Toluene	-	-	400
3	AIBN	0.09	0.2M in toluene	450
	Toluene	-	-	250

The functional monomer (MAA), crosslinker (EDGMA) and porogen (toluene) were added into three separate 1 mL GC vials. Initiator (AIBN) was then added to the mixture in the different amounts as shown in Table 3.1. The vials were briefly purged with argon, sealed and placed in an oven at 60°C for 24 h. The samples were then dried in a vacuum sample concentrator for 1 h at 50 °C.

The NIPs were then weighed, and the yields calculated.

3.1.2: Small scale MIP synthesis

Four MIP reactions were performed (Table 3.2), one for each selected pesticide template. The MIP reactions were first performed on the same scale as the NIPs described above and later scaled up, retaining the same molar ratios, so that sufficient material was available for subsequent experiments. The synthesis procedure from literature was performed at a tenth of the scale with the same molar ratios. The template molecule was subsequently further decreased to a quarter of the literature molar ratio (Kueseng *et al.*, 2009).

Table 3.2: The reagent amounts used for MIP synthesis for each of the pesticides

Reagents		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Density (g/mL)**	**Volume (µL)**
MAA (Methacrylic acid)		0.4	86.06	34.4	1.02	35.09
EGDMA (ethylene glycoldimethacrylate)		0.4	198.22	79.2	1.05	83.16
Reagents in solution		**Amount added (mmol)**	**Concentration of stock solution**	**Mass (mg)**	**Molar mass (g/mol)**	**Volume (µL)**
AIBN		0.045	0.2M in toluene		164.21	475
Toluene		-	-			225
Template		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Density (g/mL)**	**Volume (µL)**
1	Atrazine	0.025	215.68	5.4		
2	Terbuthylazine	0.025	229.71	5.7		
3	Acetochlor	0.025	269.77	6.7	1.1	7.37
4	Alachlor	0.025	269.77	6.7		

Template (pesticide), functional monomer (MAA), crosslinker (EDGMA) and porogen (toluene) were added into 1 mL GC vials. Initiator (AIBD) was then added to the mixture. The vials were briefly purged with argon and sealed. After 3 min sonication, the vials were placed in an oven at 60 °C for 24 h. The samples were then dried in a vacuum sample concentrator for 1 h.

The GC vials were weighed before the reaction was performed so that a difference in vial mass could be used to calculate the mass of the synthesised MIPs. A Soxhlet extraction was performed on the synthesised MIP containing the atrazine template. The Soxhlet was run over 2 days, for roughly 36 hours. Methanol was used as solvent and the temperature was set to 65 °C (Kueseng *et al.*, 2009).

Thereafter, the atrazine MIPs were placed in a centrifuge tube with 5 mL methanol and placed in an ultrasonic bath for 5 min. The sample was centrifuged at 3000 rpm, for 3 min. The supernatant was removed and analysed for atrazine via GC-MS analysis according to the method described in Section 3.2.

3.1.3: Batch scale MIP synthesis

MIPs were prepared on larger scale than the synthesis described in Section 3.1.2. Table 3.3 provides a summary of the synthesis procedure. To ensure enough MIP was prepared, the synthesis experiment was repeated 4 times.

Table 3.3: Batch scale MIP synthesis summary for each of the four pesticide templates

Reagent		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Density (g/mL)**	**Volume (µL)**
MAA (Methacrylic acid)		2	86.06	172	1.02	206.4
EGDMA (ethylene glycolmethacrylate)		2	198.22	396	1.05	415.8
Reagents in solution		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Concentration of stock solution**	**Volume (µL)**
AIBN		0.225	164.21	37.6	0.2 M in toluene	2 375
Toluene		-	-			1125
Template		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**		
1	Atrazine	0.025	215.68	5.4		
2	Terbuthylazine	0.025	229.71	5.7		
3	Acetochlor	0.025	269.77	6.7		
4	Alachlor	0.025	269.77	6.7		

Template (+- 0.3 mg), functional monomer (MAA), crosslinker (EDGMA) and porogen (toluene) were added into a 15 mL head space crimp cap vial. Initiator (AIBD) was then added to the mixture. The vials were purged with argon for a short time and sealed. The vials were sonicated for 3 min and placed in an oven at 60 °C for 24 h. The MIP was then dried in an oven at 60 °C overnight.

The dried MIP was transferred to centrifuge tube. A 5 mL solution of 9:1 methanol: glacial acetic acid was added and the tubes were vortexed to ensure all the polymer was suspended in solution. The

solutions were centrifuged at 3500 rpm for 5 min. The supernatant was decanted carefully, so as to disturb the sedimented MIP as little as possible. This procedure was repeated seven times.

To confirm the template was removed, 50 µL samples were taken from the supernatant decanted off each MIP to confirm the absence of template. The fractions were filtered before GC-MS analysis was performed.

The remaining MIP was then oven dried at 77.5 °C overnight to remove ethyl acetate and methanol.

This free radical polymerization reaction was repeated for terbuthylazine, acetochlor and alachlor, respectively. A NIP batch was also prepared in the same manner, except no template was added to the vial.

3.1.4: Synthesis of enhanced adsorption capacity MIPs

The aim of this synthesis procedure was to observe if the amount of template molecule added during the polymerization reaction could affect the adsorption capacity of the MIP. Enhanced adsorption capacity MIP (EAC MIP) was prepared where more template was used in the preparation steps. The molar ratios were kept identical to those described in Table 3.3, except the template was increased 4 times, from 0.025 mmol to 0.1 mmol (Table 3.4).

Table 3.4: Summary of MIP synthesis with enhanced binding capacity

Reagent		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Density (g/mL)**	**Volume (µL)**
MAA (Methacrylic acid)		2	86.06	172.12	1.02	168.7
EGDMA		2	198.22	396.44	1.05	377.6
Reagents in solution		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Concentration of stock solution**	**Volume (µL)**
AIBN		0.225	164.21	37.6	0.2M in toluene	1125
Toluene		-	-			1875
Templates		**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Density (g/mL)**	**Volume (µL)**
1	Atrazine	0.1	215.68	21.5		
2	Terbuthylazine	0.1	229.71	22.9		
3	Acetochlor	0.1	269.77	26.9	1.1	24.5
4	Alachlor	0.1	269.77	26.9		

Template (+- 0.3 mg), functional monomer (MAA), crosslinker (EDGMA) and porogen (toluene) were added into a 15 mL head space crimp cap vial. Initiator (AIBD) was then added to the mixture. The vials were purged with argon for a short time and sealed. They were then sonicated for 3 min and placed in an oven at 60°C for 24 h.

The dried MIP was transferred to centrifuge tube. A 5 mL solution of 9:1 methanol: glacial acetic acid was added to the centrifuge tube and the vials were vortexed to ensure all the polymer was suspended in solution. The tube was then centrifuged at 3500 rpm for 5 min. The supernatant was decanted carefully, so as to disturb the sedimented MIP as little as possible, and this procedure was repeated ten times to ensure all the template was removed. Then 5 mL methanol was added into the tube and it was vortexed thoroughly to remove all the acetic acid, followed by centrifugation at 3500 rpm for 5 min. The methanol was decanted and the EAC MIP was dried at 60 °C overnight before being weighed.

3.1.5: Multi-template MIP synthesis

The multi-template MIP (MTMIP) was prepared in a similar manner as the previously described polymerization procedures. The aim was to prepare a MIP that has affinity for all four selected pesticides. To work sparingly with materials and reagents, the template molar ratio described in Section 3.1.3 was halved. Table 3.5 describes the reagents used for the MTMIP synthesis.

Table 3.5: Summary of MIP synthesis for multi-template binding capacity

Reagent	**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Density (g/mL)**	**Volume (µL)**
MAA (Methacrylic acid)	2	86.06	172.12	1.02	168.7
EGDMA	2	198.22	396.44	1.05	377.6
Reagents in solution	**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Concentration of stock solution**	**Volume (µL)**
AIBN	0.225	164.21	37.6	0.2M in toluene	1125
Toluene	-	-			1875
Templates	**Amount added (mmol)**	**Molar mass (g/mol)**	**Mass (mg)**	**Density (g/mL)**	**Volume (µL)**
Atrazine	0.05	215.68	10.8		
Terbuthylazine	0.05	229.71	11.4		
Acetochlor	0.05	269.77	13.4	1.1	14.8
Alachlor	0.05	269.77	13.4		

All four templates (+- 0.3 mg), functional monomer (MAA), crosslinker (EDGMA) and porogen (toluene) were added into a 15 mL head space crimp cap vial. Initiator (AIBD) was then added to the mixture. The vial was purged with argon for a short time and sealed. The vial was briefly vortexed and sonicated for 3 min then placed in an oven at 60°C for 24 h.

The synthesised polymer was then washed with 10 mL 9:1 methanol: glacial acetic acid solution in a sonic bath for 3 min followed by vortexing and then centrifugation at 3500 rpm for 5 min, this procedure was repeated seven times. The solvent was decanted so as to disturb the sedimented

polymer as little as possible and new solvent was then added. The polymer was dried and weighed in the same manner as for the other MIPs previously discussed.

3.2: GC-MS Method

3.2.1: Introduction

It should be noted that many other samples were analysed on the GC-MS system employed, as it was not exclusively used for this study. In total two columns were used over the course of the study, as described in Table 3.6 below. First the Elite 5 MS column (5% diphenyl 95% dimethyl polysiloxane, 30 m x 0.25 mm i.d., 0.25 µm film thickness) from PerkinElmer was used then a Phenomenex ZB-5 column (5% diphenyl 95% dimethyl polysiloxane, 30 m x 0.25 mm i.d., 0.25 µm film thickness) with a 5 m guard column from Zebron was used for the pesticide analysis. The columns had equivalent phases and lengths and are the equivalents from two different suppliers. The column was changed when excessive tailing was observed that could not be fixed with simple trouble shooting procedures such as washing/replacing the inlet liner or replacing the septum. All the GC-MS analyses were done on the system described in Table 3.6.

There were no notable performance differences between the columns, except for small changes in retention time, and the 5 m guard column was found to notably extend the lifetime of the column. The columns were switched back to front, a piece of the guard column cut off, or a replacement column was installed if excessive peak tailing was observed. After the new column was installed the retention times of the analytes of interest were adjusted in the processing method and re-calibration was always necessary after performing planned preventative maintenance.

Table 3.6: GC-MS instrument setup employed for the study

Component	Make
GC	PerkinElmer Clarus 680
Detector (MS)	PerkinElmer Clarus SQ 8T
Autosampler	Mounted liquid injection autosampler from PerkinElmer
Data handling PC	HP Pro one 400- with Empower 3 for instrument control software and data processing

The mass spectrometer (MS) was calibrated and tuned with perfluorotributylamine (PFTBA) monthly unless mass inaccuracy was observed, and the MS tune was checked at least weekly. An electron ionization (EI) source was used at 70 eV.

The specific analyte mass fragments, retention time and relative abundance is summarized in Table 3.7.

Table 3.7: GC-MS analyte summary of mass fragments, retention time and relative abundance

Analyte	Mass fragments *m/z* (Da)	Retention time (min)	Isotope ratio for quantitation ions			
			m/z (Da)	Relative abundance (%)	*m/z* (Da)	Relative abundance (%)
Atrazine	200; 215	3.8	200	100	215	61.5
Terbuthylazine	214; 173	3.9	214	100	173	58.5
Caffeine Internal standard (IS)	194; 109	4.2	194	100	109	72.1
Acetochlor	59; 146; 162	4.3	146	83.9	162	69.6
Alachlor	45; 160; 188	4.4	160	37.8	188	30.4

A GC-MS run, with the MS in scan mode, was performed using the oven method described in Section 3.2.2 to find the retention times of the pesticides and analytes of interest. For acetochlor and alachlor the highest relative abundance for mass fragments have *m/z* values of 59 and 45, respectively. However, these ions were not selected for quantitation purposes as the noise and background on these lower mass ions were found to be far worse than their higher mass counterparts. The limit of detection and quantitation are thus dependent on a mass fragment with a lower intensity. Especially for alachlor this can influence the limit of detection, where the limit of detection (LOD) is defined as the concentration which has a signal to noise ratio (S/N) of 3, and the limit of quantitation (LOQ) has a S/N of at least 10.

The relative abundance for mass fragments is listed for the quantitation ions in Table 3.7. To serve as confirmation in the selected ion recording (SIR) method, the relative abundance in the peak areas for the two selected quantitation ions can be compared with values found in reference spectra. This adds confidence to the detection of the pesticide and confirms the absence of interferences for quantitation purposes. Both the quantitation ions need to be present and the relative abundance must typically be within 20% of the expected ratio before a positive match is given for the pesticide. The ion ratio can vary because of interferences or because the MS is tuned differently. Turbo mass, which is used to operate the MS has an auto tune function that adjusts the multiplier voltage as well as the low and high mass resolution to obtain adequate intensity and resolution. The tune file parameters can be adjusted manually to try and obtain better resolution or intensity. Eventually the ion source and pre-quads must be cleaned if the multiplier voltage gets excessively high, normally a voltage

around 2000 V indicates ion source cleaning is necessary. If an optimal MS mass resolution cannot be achieved, the pre-quads must be cleaned.

The oven time was optimized by altering ramp rates and starting temperature so that optimal separation could be achieved in minimal time. The same GC method was then used with a selected ion recording (SIR) method on the MS to enhance detection limits and selectivity.

Working standard (WS) preparation steps as used to develop the GC-MS method:

The four pesticide standards were supplied at 100 µg/mL concentration in 1 mL of methanol. The four pesticide reference standards were mixed, 200 µL of each, resulting in a 800 µL working standard (WS) with each pesticide at 25 µg/mL concentration in methanol. A solution containing 25 µL (25 ug/mL pesticide WS) and 25 µL (25 µg/mL caffeine) was prepared. The concentration for all the analytes were 12.5 µg/mL.

3.2.2: GC-MS Instrumental parameters

Table 3.8 displays the oven temperature program utilised for the analysis of the four selected pesticides. The program consists of four phases, starting at 80 °C, the temperature is quickly ramped up to 220 °C. The increase in temperature is then slowed over the retention times of the analytes and then ramped quickly to 300 °C, 20 °C under the maximum column temperature. The temperature is held at 300 °C for 1 min to allow any compounds still on the column time to bake out. The total run time was 8.8 min.

Table 3.8: The GC oven program employed for the study

Phase	Ramp (°C/ min)	Temperature (°C)	Hold time (min)
1	-	80	0
2	50	220	0
3	10	260	0
4	40	300	1

Other instrumental parameters included a 250 °C injector temperature, 1 µL injection volume in spitless mode, with a double taper goose neck liner (PerkinElmer). After 0.2 min, the split flow was altered to 15 mL/min (split ratio: 15:1). The column flow rate was 1.0 mL/min (constant flow) with helium as carrier gas.

Scan method:

The transfer line and MS source temperature were both set to 200 °C. The MS functions are described in Table 3.9.

Table 3.9: MS function events for scan mode operation of the GC-MS

Time (min)	Function	Scan range *m/z* (Da)
0 - 3.5	Solvent delay	N/A
3.5 - 5	Scan	50 - 300
6 - 8.8	Solvent delay	N/A

The scan mode on the MS allows for rapid scanning of masses in a mass range. The collected data is then referred to as a total ion current (TIC) chromatogram. The TIC represents the summed intensity across the entire range of mass to charge ratio, in this case 50-300 Da, being detected at every point in the analysis. The data analysis software allows for the extraction and viewing of selected ion chromatograms. The mass spectra can also be exported to the NIST MS Search 2.2 software package, that allows the experimental mass spectra to be compared to mass spectra stored in the NIST library. This is useful to identify unknown peaks in the spectra or to confirm retention times of expected analytes using a specific GC instrument method. If retention times shift because of column degradation or matrix effects, this is a very useful tool to confirm the identity of analytes. The solvent delay function on the MS switches the filament of the EI ion source off and no ionization takes place. The purpose of the solvent delay is to spare the filament and ion source from overworking when an extremely high intensity component, such as the solvent, exits the GC column. Using the solvent delay can lengthen the lifetime of the filament and prevent the ion source from becoming very dirty too quickly.

SIR method:

Using the information obtained from the total ion current (TIC), a selected ion recording (SIR) MS method was set up. SIR is superior in terms of sensitivity, as the signal to noise ratio is vastly improved. A drawback of using SIR is that later data mining is not possible as all the MS data is no longer available. For commercial laboratories doing routine analysis, data mining is usually not employed. This study also has a targeted approach on four selected pesticides, thus SIR was employed. SIR allows for lower limits of detection and quantitation. It is also preferred for quantitation because more data points can be collected over a peak, since fewer mass channels are monitored, thus allowing for better repeatability and accuracy when compared to a TIC approach. The SIR MS method is summarized in Table 3.10.

Table 3.10: MS function events for SIR mode operation

Time (min)	Function	Mass ratios (*m/z*) recorded
0 - 3.5	Solvent delay	N/A
3.5 – 4.9	SIR window 1	43; 58; 173; 200; 214; 215
4.8 - 6	SIR window 2	45; 59; 109; 146; 160; 162; 188; 194
6 - 8.8	Solvent delay	N/A

Depending on the manufacturer, selected ion recording (SIR) can also be referred to as selected ion monitoring (SIM).

3.2.3: Calibration

A suitable SIR method was developed for the detection of the four selected pesticides as described in Section 3.2.2.

The maximum residue limit (MRL) for atrazine, terbuthylazine, acetochlor and alachlor is 0.05 mg/kg (or 50 ppb) on South African crops (Department of National Health and Population Development, 2012). Having the calibration curve around 10 ppm instead of a lower concentration results in a more robust and repeatable calibration curve since it is less affected by background noise and instrumental drift. In the sample preparation method, a pre-concentration step was thus required to obtain the desired concentration range. Both SPE and evaporation, utilising a vacuum sample concentrator, can be used to achieve this. Using an internal standard (IS) adds further robustness to the method since not only peak area is brought into consideration but rather a peak ratio of analyte to IS. The weighting for all curves was set to 1/X with a linear fit not forced through zero.

Two concentrations were selected above and below 10 ppm respectively, resulting in a 5-point calibration curve (Table 3.11).

Table 3.11: Five point calibration curve preparation indicating the amounts of standard, internal standard and methanol solution required to obtain the desired concentrations

Calibration point	1	2	3	4	5
Pesticide WS (µL) at 25 µg/mL	5	10	20	30	40
Caffeine IS (µL) at 25 µg/mL	10	10	10	10	10
Methanol (µL)	35	30	20	10	0
Pesticide concentration (µg/mL)	**2.5**	**5**	**10**	**15**	**20**
Caffeine concentration (µg/mL)	**5**	**5**	**5**	**5**	**5**

Calibration sample preparation procedure

The indicated volume (Table 3.11) of 25 µg/mL pesticide working solution and 10 µL of 25 µg/mL caffeine solution were mixed in a GC vial containing a 200 µL insert. Methanol was then added to a volume of 50 µL. The calibration samples were vortexed and run on the GC-MS system in quadruplicate for statistical analysis.

3.2.4: Silanization and deactivation

Silanization or deactivation of the quartz double taper goose neck inlet liners was found to be a very important step in this study, especially for the analysis of atrazine, which exhibited excessive peak tailing when silanization was not done properly or not often enough. Analytes with hydroxyl, amino or phosphate groups are known to undergo interactions and reactions with glass and metal surfaces, especially under the heated gas phase conditions of GC (Kwon *et al.* 2012).

Liners are made up of borosilicate or quartz glass. The surface contains active sites, such as silanol and siloxane groups (Klee 2005). These silanol groups on the glass were deactivated by submerging the liner in a 10% DMDCS toluene solution for at least 30 min. The liner was then thoroughly rinsed with methanol and dried before use.

Since the instrument was used for the analysis of other samples, it was found the liners needed to be cleaned and silanized regularly to maintain good chromatography and baseline separation of the analytes. From time to time it was found to be necessary to also cut off a portion of the inlet or guard side of the column (a portion of about 30 to 40 cm was found to be adequate). The washing and deactivation of the inlet liner and the occasional cutting of column ensured optimal chromatography was maintained.

3.2.5: Matrix effects

Besides the matrix effects from the sample, solvent or diluent effects were also observed during method development. Peak tailing was observed in aqueous samples, especially for the triazine pesticides, whilst the chloroacetamide pesticides were largely unaffected in terms of peak shape. This observation was made in an experiment where the aqueous loading or wash fractions were analysed during a SPE elution procedure. The drying and subsequent reconstitution of samples with methanol was thus found to be of paramount importance to obtain suitable chromatography and baseline separation between the triazine pesticides. The procedure used in this regard was as follows: Samples were transferred to open GC vials and placed inside the disk of the CHRIST vacuum concentrator. For aqueous samples, the oven temperature was set at 55 °C and the chiller temperature was set to 2 °C.

The chiller passes liquid through the condenser, to prevent the condenser from freezing up and when many aqueous samples are evaporated at the same time the temperature cannot be set below 0 °C. Aqueous samples were found to take a long time to evaporate, up to an hour per mL. If samples of more than 2 mL were to be evaporated, a GC vial containing 2 mL sample was placed in the evaporator for an hour. After full evaporation, an additional 2 mL of the remaining sample can be added to the same GC vial and the evaporation process continued. This process can be repeated until no more sample remains to be evaporated, after which 50 µL methanol was added to the GC vial and vortexed thoroughly. The solution was then transferred to a 200 µL GC vial insert before capping the vial.

3.3: Adsorption capacity determination

Template extraction efficiency and target pesticide adsorption capacity were determined using Equations 1 and 2, respectively.

Equation 1:

$$Extraction\ efficiency\ (\%) = \frac{(C_0 - C_e)}{C_0} \times 100$$

Equation 2:

$$Adsorption\ capacity\ (mg/g) = \frac{(C_0 - C_e)V}{W}$$

Where C_0 represents the initial concentration (mg/L) before adsorption and C_e the final concentration (mg /L) of the target compound remaining in solution after adsorption. The volume of the solution is represented by *V*, in litres, and W represents the mass of the polymer in grams.

3.3.1: MIP adsorption capacity determination

From literature, the binding capacity of the MIP is expected to be about 1 mg/g (Kueseng *et al.*, 2009). This can be used to determine relevant experimental conditions at concentrations that can be accurately determined by GC-MS. For the calibration curve on the GC-MS, the highest concentration is 20 µg/mL, this can be C_0. The analytical balance has an uncertainty of measurement of +- 0.1 mg. Very little polymer can thus be weighed, saving on experimental time and cost.

If, for example 30 mg of polymer is weighed, C_0 is 20 µg/mL, the adsorption capacity is 1 mg/g and the volume is 4 mL then $C_e$can be calculated as follows:

$$Adsorption\ capacity\ = \frac{(C_0 - C_e)V}{W}$$

$$Adsorption\ capacity\ \times W = (C_0 - C_e)V$$

$$\frac{Adsorption\ capacity\ \times W}{V} = C_0 - C_e$$

$$C_e = C_0 - \frac{Adsorption\ capacity\ \times\ W}{V}$$

$$C_e = 20\ \mu g/ml - \frac{1\ mg/g\ \times\ 30\ mg}{4\ mL}$$

$$C_e = 12.5\ \mu g/mL$$

A final C_e concentration 12.5 µg/mL is perfectly within the range of the calibration curve; the suggested values are thus fit for purpose.

Experimental procedure:

A known amount of washed and dried polymer (MIP or NIP), close to 30 mg, was weighed into a centrifuge tube. Milli Q grade water (3960 µL) was then added to the centrifuge tube.

It should be noted the polymer was found to be quite hydrophobic and could float on top of the water meniscus. It worked best to let the water run slowly down the side of the tube to cover the loose polymer at the bottom.

The selected pesticide was then added, 40 µL of 2 mg/mL standard solution, to provide a concentration of 20 µg/mL in a total of 4 mL. The tube was then sealed and vigorously mixed to allow the polymer to come into contact with the entire solution. The vial was both vortexed and sonicated for 3 min and 20 min, respectively. The mixture was then centrifuged for 5 min at 3500 rpm.

Samples were taken from the centrifuge tube supernatant and filtered through a 0.24 µm syringe filter. The sample was then dried via vacuum concentration and reconstituted with 50 µL methanol containing 5 µg/mL internal standard before analysis was performed by GC-MS.

The calibration detailed in Table 3.12 was done specifically for the quantitation of the pesticide in the aqueous solution where the highest point of the calibration is 20 µg/mL. A pesticide mixture working standard (WS) of 25 ug/mL of each selected pesticide in methanol was prepared, along with an internal standard (IS) of 25 ug/mL caffeine in methanol.

Table 3.12: Calibration curve preparation summary for adsorption capacity determination

Calibration point #	**1**	**2**	**3**	**4**	**5**
WS (µL)	5	10	20	30	40
IS (µL)	10	10	10	10	10
MeOH (µL)	35	30	20	10	0
[IS] (µg/mL)	5	5	5	5	5
[WS] (µg/mL)	2.5	5	10	15	20

3.3.2: MIP triazine and MIP chloroacetanilide pesticide class adsorption capacity

The use of dummy templates, referred to in Section 2.7.1 suggest that the atrazine MIP may also adsorb terbuthylazine and vice versa. The same would naturally apply for acetochlor and alachlor and their complementary MIPs. The adsorption capacity was thus determined for terbuthylazine on the

atrazine MIP and vice versa. The adsorption capacity for alachlor was also determined on the acetochlor MIP and vice versa. Each experiment was repeated three times for statistical purposes in the same manner as described in Section 3.3.1.

3.3.3: EAC MIP adsorption capacity

The adsorption capacity for the enhanced adsorption capacity (EAC) MIP (from Section 3.1.4) was determined for each separate pesticide and its complimentary MIP. The same calibration curve and methodology was used as in previous experiments (refer to Section 3.3.1). Again, each experiment was repeated three times for statistical analysis.

3.3.4: MTMIP adsorption capacity

The adsorption capacity of the multi-template (MT) MIP, synthesised in Section 3.1.5, was determined for each separate pesticide on the same MTMIP. Each experiment was repeated three times for statistical analysis. The same calibration curve and methodology was used as in previous experiments, refer to Section 3.3.1.

3.4 : MIP characterization

3.4.1: SEM

A Zeiss Evo MA10 electron microscope (supplied by Carl Zeiss (Pty) Limited, South Africa) was used to take scanning electron microscopy (SEM) images using conductive double-sided adhesive carbon tabs to mount samples. The polymers are directly mounted onto the carbon stub, and no coating was performed before SEM imaging. The adhesive carbon disk provided the conductivity necessary for SEM imaging, as the polymer particles themselves are not conductive. Electron high tension (ETH), also referred to as acceleration voltage, represents the effective voltage on the electron along its straight line of travel. A ETH voltage of 4.0 to 5.0 kV was used in this study.

3.4.2: FTIR

A PerkinElmer Spectrum Two with ATR source (supplied by PerkinElmer South Africa (Pty) Ltd) was used to obtain FTIR spectra of the synthesised MIPs and NIP. Technical grade 70% isopropanol (Honeywell, North Carolina, United States) and soft, lint free Kimwipes® (Kimtech™ of Kimberly-Clark, United Sates, Texas) were used to clean the ATR source and compression boot before and after each sample was mounted. After the source dried, a blank or background spectrum was obtained. The sample was then placed on the ATR source and compressed under the compression boot.

3.5: SPE cartridge packing

3.5.1: Polymer size fractionation

Size fractionation of the MIPs and NIPs were performed by wet sieving of the polymer. Empty SPE cartridges with frits (Chromabond by Machery-Nagel) were obtained from Separations. The 6 mL polyethylene cartridges come with an installed 20 µm frit at the bottom of the cartridge. Separate 20 µm frits are supplied that can be installed above the sorbent after it has been packed into the cartridge.

Because the empty SPE tubes come with 20 µm frits, a 24 µm stainless steel sieve from Labotec was selected for the smaller sieve for MIP size fractionation purposes. This prevented sorbent from coming through the 20 µm frit after the molecularly imprinted solid phase extraction (MISPE) cartridge had been packed. To prevent channelling through the sorbent bed, a 53 µm stainless steel sieve was selected to be the larger sieve used for size fractionation and the sieves fitted onto one another to create a cascade of sieves.

The polymer particles were washed through the sieve with deionised water. The water and the particles smaller than 24 µm were collected in a waste container for disposal. The particles on the top of the 53 µm sieve were ground with an agate mortar and pestle and passed through the sieves again to ensure a maximal fraction of particles with a diameter of 24-53 µm was collected. The grinding and sieving process was repeated several times. The remaining >53 µm fraction was then collected and placed in a labelled vial for potential future use. The collected 24 to 53 µm size fraction was placed in a desiccator and dried under vacuum, after which it was used to pack the MISPE cartridges (Kueseng *et al.*, 2009).

It is noteworthy to mention that after the polymers were ground for sieving, more pesticide template could be washed out, thus suggesting that new imprinted sites were exposed in the polymer. It is thus also necessary to rinse the MIPs further after grinding before MISPE experiments can be performed. A 9:1 methanol to glacial acetic acid mixture was used to remove the pesticide template from the MIP (Kueseng *et al.*, 2009). The sonication and subsequent centrifugation washing procedure was previously described in Section 3.1.3 with MIP synthesis. This washing procedure was repeated before the MIPs were packed into the cartridges.

3.5.2: SPE cartridge packing with MIPs and NIPs

The same procedure was followed for the packing of all MIP and NIP SPE cartridges. The procedure is based on literature, typically MISPE cartridges are packed with 100 to 200 mg MIP sorbent, compressed between two frits (Wang *et al.*, 2015).

It was found that washing the size fractionated polymers with methanol before drying resulted in less conglomeration or clump formation of the polymer and quicker drying in comparison to water. After the collected 24 to 53 µm size fraction was fully dried, it was mixed thoroughly in a centrifuge tube by vortexing. The polymer was then weighed into the empty SPE cartridge, which was subsequently placed on the vacuum manifold with the tap closed. Methanol was added to submerge the polymer completely. The cartridge was flicked and tapped to ensure no air was trapped between the polymer particles. The second frit was then pressed down into the cartridge until it tightly pressed against the packed polymer. The volume of a 3 mm bed length packed MISPE cartridge of radius 6.5 mm was calculated to be 0.4 mL. The methanol was then sucked out of the cartridge using the vacuum. Wet packing proved to be far superior to dry packing in terms of repeatability in the experimental results and channelling through the column.

It was also found to be necessary to store the MISPEs wet, not allowing them to dry, in order to prevent channelling. When the MISPE dried, visible cracks formed rendering it ineffective for trapping pesticides. It was found that the MISPE could be recovered after it dried by repeated wetting and recompression although this was time consuming.

Before the packed MISPEs were used, methanol was passed through the cartridges. This was done both to condition the sorbent bed and to ensure all the pesticide template had been removed. These methanol fractions were analysed by GC-MS to ensure the absence of template pesticide.

3.6: Preliminary SPE method

These SPE experiments aimed to demonstrate that the synthesised polymers could function under ideal conditions. There are some differences between the C18 SPE and the MISPE methods, as shown in Figure 3.1.

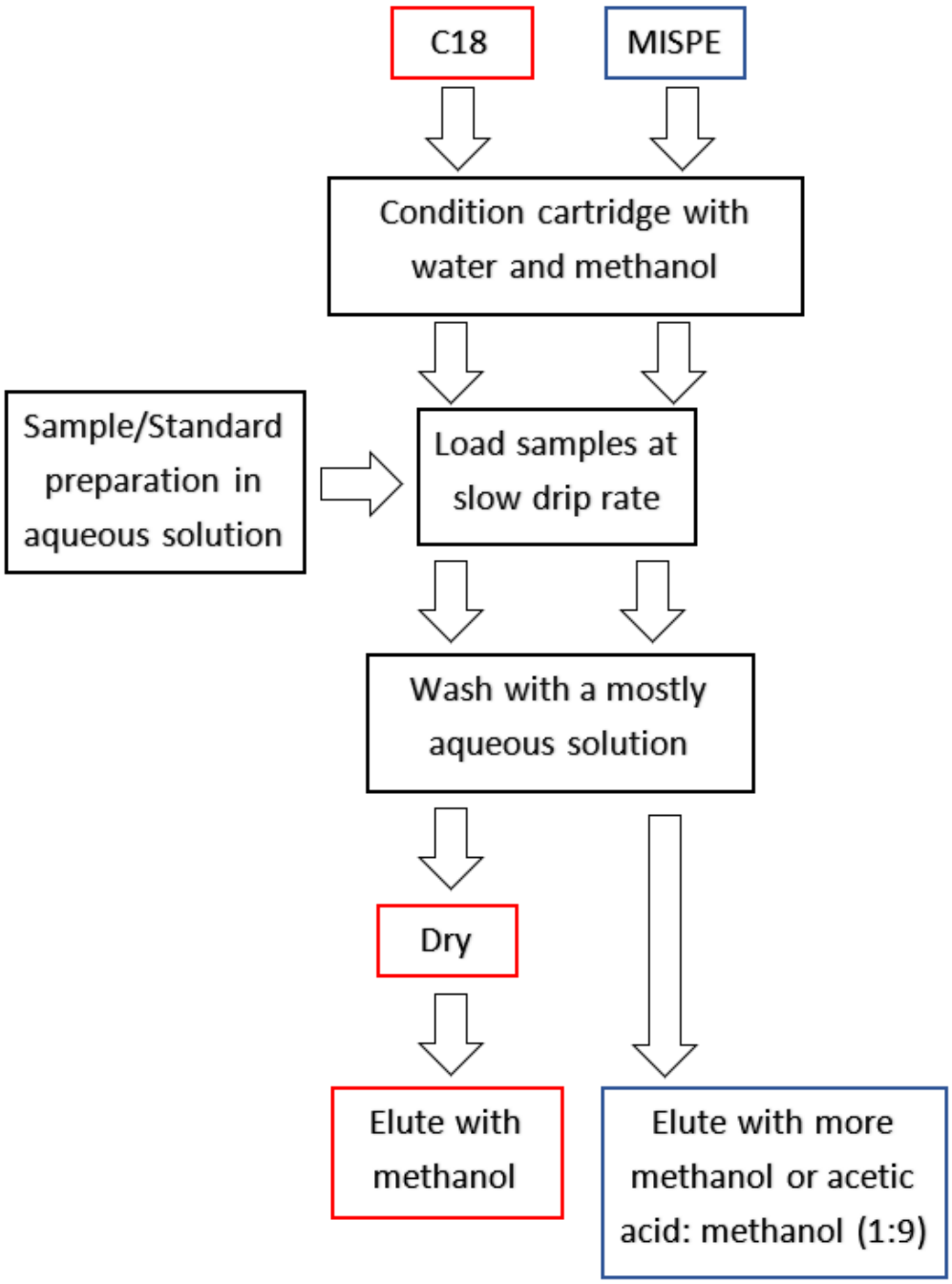


Figure 3.1: Flow diagram depicting the core differences between the C18 SPE and MISPE methods

3.6.1: C18 SPE method

Strata C18-E SPE cartridges (Figure 2.20) with a bed volume of 100 mg in a 3 mL syringe were used as a reference to the MISPE. The sorbent is non-polar, meaning a polar solvent must be used as the loading fraction so that pesticides will have a higher affinity for the sorbent than the solvent. A less polar elution solvent will then be needed to elute the pesticides from the SPE cartridge, while the least polar matrix interferences remain adsorbed to the sorbent. Methanol was thus used as elution solvent (Ma *et al.,* 2003).

3.6.1.1: C18 SPE experiment with higher concentrations of atrazine, acetochlor, terbuthylazine, and alachlor.

SPE Method

The C18 SPE sorbent was conditioned with 1 mL methanol and then 2 mL of water. To prepare the loading fraction, 50 µL of 2 mg/mL atrazine, acetochlor, terbuthylazine, and alachlor in methanol were mixed and diluted to 3 mL with MilliQ water. The concentration of each of the pesticides in the loading fraction was thus 33 µg/mL. Before the SPE dried after conditioning, the diluted pesticide sample was loaded onto the cartridge at a slow drip rate of approximately 0.5 mL/min. The SPE cartridge was then washed with 3 mL of Milli Q water at approximately 0.8 mL/min. The C18 sorbent was dried under a vacuum of 50 kPa for 30 min. The pesticides were eluted using 2 mL methanol fractions, 3 times. A theoretical 100% extraction efficiency gives a concentration of 50 µg/mL for each pesticide, 50 µL subsamples of each fraction were dried and reconstituted with 50 µL methanol containing 5 µg/mL caffeine as internal standard. Samples of the loading wash and 3 elution fractions were injected (1 µL injection volume) into the GC-MS for analysis with the previously developed GC-MS method (Section 3.2). An injection split ratio of 3:1 was used to bring the sample concentration into the calibration range. The Empower 3 software mathematically corrects for the split by including a multiplier of 3.

Control sample preparation

25 µL of 2 mg/mL atrazine, acetochlor, terbuthylazine, and alachlor in methanol was each added to 900 µL of methanol. The concentration of the pesticides in the reference was 50 µg/mL, equal to the theoretical 100% elution concentration of the SPE method. A 50 µL subsample was dried and reconstituted with methanol containing 5 µg/mL caffeine. An injection split ratio of 3:1 was used to bring the control sample concentration into the calibration range. This was done to verify that the Empower 3 software mathematically corrected for the split.

3.6.1.2: C18 SPE experiment at a lower concentration of atrazine and for a cannabis matrix

Cannabis reference sample

About 20 mg of cannabis flower samples (Figure 3.2) were weighed after mixing and blending.

Figure 3.2: Examples of cannabis flower or bud samples that were spiked in this experiment

Cannabis flower sample (approximately 20 mg) was weighed into a test tube, then 50 µL of the 20 µg/mL atrazine in methanol solution was added giving a 0.05 µg/mg concentration of atrazine on the cannabis flower. (This is 0.05 mg/g concentration, which is quite far above the 0.05 mg/kg South African MRL but was used for test purposes). The samples in the test tubes were air dried to remove the methanol before continuing. Water (2 mL) was added to each test tube resulting in a 10 mg/mL slurry, with an atrazine concentration of 0.5 µg/mL. The sample was vortexed thoroughly and placed in the ultrasonic bath at 40 °C for 20 min. The samples were then allowed to cool to room temperature before filtering with a Sartorius 0.45 µm PTFE syringe filter. The solution was then loaded onto the SPE cartridge, and five replicates were performed for statistical analysis purposes.

C18 SPE method

The C18 SPE cartridge was conditioned with 1 mL methanol and then 2 mL of water. Before the SPE dried, the diluted sample was loaded onto the cartridge at a slow drip rate of approximately 0.5 mL/min. It was observed that the colour of the loading phase was a hazy yellow, whilst after it was passed through the SPE cartridge the colour was a clear yellow. The SPE cartridge was then washed with 3 mL water, at approximately 0.8 mL/min. The wash fraction was also slightly yellow in colour. The sorbent was dried under a vacuum of -50 kPa for 30 min. The atrazine was eluted using 2 mL of methanol, at 0.5 mL/min, in theory resulting in 0.5 µg/mL sample. The methanol fraction was clear and colourless. A 1 mL sample of the elution fraction was taken, dried on the vacuum sample

concentrator at 40 °C, and reconstituted with 50 µL methanol containing 5 µg/mL caffeine IS before injection into the GC-MS using the method described in Section 3.2. A multiplier of 2 was added to the run list, to compensate for only taking 1 mL of the 2 mL elution fraction. A dilution factor of 0.05 (50/ 1000) was also added to the run list to compensate for the vacuum concentration and reconstitution of 1 mL to 50 µL. An injection volume of 1 µL was used.

Control sample preparation

The desired 20 µg/mL atrazine solution was prepared by diluting a 2 mg/mL working solution 100 times. 50 µL of 20 µg/mL atrazine in methanol was added to 1.95 mL of methanol, to match the 2 mL methanol used in the elution step of the SPE method. The concentration of atrazine in this solution was 0.5 µg/mL. This was done in a GC vial and 1 mL of the control sample was dried and reconstituted in the same manner as the samples. The same multipliers and dilution factors were added to the Empower software before injection into the GC-MS for analysis using the method described in Section 3.2.

3.6.2: MISPE and NISPE method

Molecularly imprinted solid phase extraction (MISPE) cartridges were packed as described in Section 3.5. Better results were obtained when the MIP SPE was packed to a similar height in comparison to the C18 SPE. Thus, rather than weighing out 100 mg of polymer to match the mass of C18 sorbent it was found that approximately 175 mg of MIP was required to reach the same height after compression. The synthesised polymer can be compressed to a smaller volume, when applying pressure during the packing procedure. It was found that packing to just below double the desired height (which relates to roughly 175 mg of synthesised polymer) before pressing down the second frit resulted in a near perfect match in the packed sorbent height when compared to the C18 sorbent height. It should be noted the MN tubes used for MISPE packing have a wider diameter than the Phenomenex C18 SPE cartridges.

The MISPE method followed was similar to the C18 reference method except for the drying step, refer to Figure 3.1. It was found that the synthesised polymer shrank in size and the packing cracked forming channels in the sorbent bed if it was dried. This severely hampered the retention of the pesticide on the sorbent. Because the drying step was left out, it was found necessary to use two 1.5 mL methanol elution fractions to elute all the pesticide from the SPE cartridge. The first elution fraction also dried a lot slower than the second in the vacuum concentrator, as there was evidently still water trapped in the sorbent bed before elution.

MISPE/NISPE method:

The MISPE/NISPE cartridge was conditioned with 1mL methanol and then 2 mL of water. The loading fraction was prepared by diluting 25 µL of 25 µg/mL pesticide in methanol to 3 mL with MilliQ water to a final concentration of 0.208 µg/mL. Before the MISPE/NISPE dried, the diluted pesticide sample was loaded onto the cartridge at a slow drip rate, approximately 0.5 mL/min. The MISPE/NISPE cartridge was then washed with 3 mL of water at approximately 0.8 mL/min. The pesticides were eluted using two fractions of 1.5 mL methanol. (This was done collect the elution fractions in 2 mL GC vials that can be placed in the sample concentrator for drying). A theoretical 100% extraction efficiency gives a concentration of 0.416 µg/mL for each separate pesticide. All the fractions where dried via vacuum concentration and reconstituted with 50 µL of 5 µg/mL caffeine in methanol giving a total volume of 50 µL. The theoretical concentration was now 12.5 µg/mL for each pesticide.

An NISPE was performed alongside each MISPE to be used as a reference.

3.7: Wash solvent optimization

In Section 3.6 only water was used as a wash solvent. Although very good recoveries were observed, to use the MISPEs as a useful tool in sample preparation some optimization is required to remove as much background and matrix interferences as possible. In order to accomplish sample preparation steps, such as clean-up and concentration, the optimal wash solvent or mixture should be employed.

Several wash solvents are reported in literature. For the most part, methanol and acetic acid solutions are used to remove the template from the MIP after synthesis and for SPE purposes (refer to Table 2.10) where a 10% glacial acetic acid: 90% methanol solution is very popular. It has been reported in literature that increasing the acetic acid concentration (from 6 % to 30%) in an acetonitrile: acetic acid mobile phase for a MIP packed HPLC column, the retention of the template was found to decrease. This observation was used to support the theory that retention of triazine pesticides was mainly due to hydrogen bonding (Matsui *et al.*, 2000). The primary interactions between the MIP and template are hydrogen bonds and electrostatic interactions, however the use of carboxylic acid allows for the easier removal of the template (Matsui *et al.,* 2000).

A similar MIP, prepared from MAA and EDGMA, involved the use of hexane as a wash solvent and methanol as the elution solvent, with recoveries of > 80.2% for acetochlor, alachlor and some other chloroacetanilide pesticides (Wang *et al.*, 2015).

A new approach was tried in this study, where the wash solvent was altered to a methanol gradient to find the optimal methanol concentration to be employed as the wash solvent. A C18 SPE methanol: water gradient was also performed to be used as a reference to the synthesised MIPs.

3.7.1: C18 SPE methanol: water wash solvent gradient

A relatively concentrated solution (not at a relevant environmental or MRL concentration) was prepared. This was done so that the 3 mL fractions containing mostly water could be analysed without drying the entire sample, but rather a small sub-sample thereof, as this would have taken a very long time given the available vacuum concentrator and how slowly aqueous samples evaporate.

The loading fraction was prepared by adding 25 µL of 2 mg/mL of each of the pesticide solutions in methanol to water to a total volume of 3 mL. The concentration of the loading fraction was thus 16.67 µg/mL.

The C18 SPE cartridge was conditioned using 3 mL of methanol, followed by 3 mL of Milli Q water. The 3 mL diluted loading fraction was then passed through the SPE cartridge at a slow flow rate of +-0.5 mL/min. Table 3.13 was used to prepare wash solvent fractions with an increasing methanol gradient. All 15 fractions, including the loading sample, had a total volume of 3 mL and were passed through the cartridge at a slow drip-rate. The more aqueous fractions needed lower/stronger vacuum pressure to pass through the C18 SPE cartridge. The later fractions, containing more methanol, passed through the C18 SPE cartridge almost without vacuum.

Table 3.13: Methanol: water gradient making up 15 fractions in a gradient from 3.3% to 80% methanol for C18 SPE wash solvent optimization

Fraction #	Methanol (%)	Water (%)	Methanol (µL)	Water (µL)
1 (Load)	3.3	96.7	100	2900
2	10	90	300	2700
3	15	85	450	2550
4	20	80	600	2400
5	25	75	750	2250
6	30	70	900	2100
7	35	65	1050	1950
8	40	60	1200	1800
9	45	55	1350	1650
10	50	50	1500	1500
11	60	40	1800	1200
12	65	35	1950	1050
13	70	30	2100	900
14	75	25	2250	750
15	80	20	2400	600

A 50 µL sample was taken from each of the fifteen 3 mL fractions and dried via the vacuum concentrator. The theoretical 100% elution efficiency is 16.67 µg/mL for the 3 mL fractions, if all the pesticide was to elute in a single fraction. Reconstitution was done with 50 µL methanol containing 5

µg/mL caffeine internal standard as for the calibration curve, therefore the theoretical concentration was thus 16.67 µg/mL. The samples were then analysed by GC-MS utilizing the previously developed method in Section 3.2.

3.7.2: NIP methanol: water wash solvent gradient

It was found necessary to start at a much lower methanol concentration for the NISPE, relative to the C18 SPE cartridge. The same amount of standard was used, although to attain a 1% methanol concentration a total volume of 10 mL was prepared for the loading fraction. The subsequent wash fractions were prepared in the 3 mL fractions, to allow for comparability with the C18 SPE experiment.

The loading fraction was prepared by adding a 25 µL methanol solution containing 2 mg/mL of each of the four pesticides to 9.9 mL water, to give a total volume of 10 mL. The concentration for the loading fraction was thus 5 µg/mL.

The NISPE cartridge was conditioned by passing 3 mL of methanol through the sorbent, followed by 3 mL of Milli Q water through the cartridge at a slow drip rate. The 10 mL diluted loading fraction was then passed through the SPE at a flow rate of +-0.5 mL/min. Table 3.14 was used to prepare wash solvent fractions with an increasing methanol gradient. All 17 fractions, excluding the loading sample, had a total volume of 3 mL and were passed through the NISPE cartridge at a slow drip-rate. The more aqueous fractions needed a lower vacuum pressure to pass through the NISPE cartridge than the higher methanol fractions.

Table 3.14: Methanol: water gradient making up 17 fractions in a gradient from 1% to 30% methanol for NISPE wash solvent optimization

Fraction #	Methanol (%)	Water (%)	Methanol (µL)	Water (µL)
1 (Load)	**1**	99	100	9900
2	**2**	98	60	2940
3	**4**	96	120	2880
4	**6**	94	180	2820
5	**8**	92	240	2760
6	**10**	90	300	2700
7	**12**	88	360	2640
8	**14**	86	420	2580
9	**16**	84	480	2520
10	**18**	82	540	2460
11	**20**	80	600	2400
12	**30**	70	900	2100
13	**40**	60	1200	1800
14	**50**	50	1500	1500
15	**60**	40	1800	1200

Fraction #	Methanol (%)	Water (%)	Methanol (µL)	Water (µL)
16	**70**	30	2100	900
17	**80**	20	2400	600

A 50 µL sample was taken from each 3 mL fraction and dried. The theoretical 100% elution efficiency was 16.67 µg/mL for the 3 mL fractions and 5 µg/mL for the 10 mL loading fraction. Subsequently 150 µL of the loading fraction was dried so it would have a concentration of 15 µg/mL after reconstitution if all the pesticide were to elute in the loading fraction. Reconstitution was done with 50 µL methanol containing 10 µg/mL internal standard as for the preparation of the calibration curve. The samples were then analysed by GC-MS utilizing the previously developed method in Section 3.2.

3.7.3: Triazine MIPs methanol: water wash solvent gradient

From the NIP experiment (Section 3.7.2) it was found that the bulk of atrazine pesticides began eluting after 20% methanol, whilst the terbuthylazine eluted only later. The aim of this experiment was to determine if there was a significant difference between the triazine MIPs, compared to the NIP, with regards to how the template molecules elute with an increase in methanol concentration.

The following procedure was used to determine the optimal wash solvent to be used for the triazine MIPs. The experiment was performed separately for each triazine pesticide on its own MISPE to ensure competition for imprinted cavities between atrazine and terbuthylazine was not a factor.

The loading fraction was prepared by adding 25 µL methanol solution containing 2 mg/mL of the specific triazine pesticide to 2975 µL water, to a total volume of 3 mL. The concentration for each separate loading fraction was thus 16.67 µg/mL.

The triazine MISPE cartridges were conditioned by passing 3 mL of methanol, followed by 3 mL of Milli Q water at a slow drip rate through each of the triazine MISPE cartridges. The 3 mL diluted loading fractions were then passed through the MISPE cartridges at a slow flow rate of +-0.5 mL/min. Table 3.15 was used to prepare wash solvent fractions with an increasing methanol gradient. All 17 fractions, including the loading sample, had a total volume of 3 mL and were passed through the MISPE cartridges at a slow drip-rate. The more aqueous fractions passed through the sorbent with more difficulty compared to the fractions containing more methanol, the vacuum pressure was adjusted accordingly for each fraction to achieve the desired drip rate.

Table 3.15: Methanol: water gradient making up 17 fractions in a gradient from 0.8% to 80% methanol for triazine MISPE wash solvent optimization

Fraction #	Methanol (%)	Water (%)	Methanol (µL)	Water (µL)
1 (Load)	**0.83**	99.167	25.0	2975
2	**10**	90	300	2700
3	**12**	88	360	2640
4	**14**	86	420	2580
5	**16**	84	480	2520
6	**18**	82	540	2460
7	**20**	80	600	2400
8	**22**	78	660	2340
9	**24**	76	720	2280
10	**26**	74	780	2220
11	**28**	72	840	2160
12	**30**	70	900	2100
13	**40**	60	1200	1800
14	**50**	50	1500	1500
15	**60**	40	1800	1200
16	**70**	30	2100	900
17	**80**	20	2400	600

A 50 µL sub sample was taken from each 3 mL fraction and dried. The theoretical 100% elution efficiency was 16.67 µg/mL for the 3 mL fractions. Reconstitution was done with 50 µL methanol containing 5 µg/mL caffeine internal standard, the same concentration as in the calibration curve. The samples were analysed by GC-MS utilizing the previously developed method in Section 3.2.

3.7.4: Chloroacetamide MIPs methanol: water wash solvent gradient

From the NIP experiment (Section 3.7.2), it was found that a significant concentration of chloroacetamide pesticides started eluting after just 2% methanol. The aim of this experiment was to determine if there was a significant difference for the chloroacetamide MIPs, compared to the NIP, with regards to the elution of the template molecules with an increase in methanol concentration.

The following procedure was used to determine the optimal methanol: water wash solvent to be used for the chloroacetamide MIPs. The experiment was performed separately for each chloroacetamide pesticide on its own MISPE to ensure competition for imprinted cavities between acetochlor and alachlor was not a factor.

The loading fraction was prepared by adding 25 µL of 2 mg/mL of each of the chloroacetamide pesticides in methanol to water, to a total volume of 3 mL. The concentration for both acetochlor and

alachlor in the loading fractions was thus 16.67 µg/mL. Each chloroacetamide MISPE cartridge was conditioned by passing 3 mL of methanol, followed by 3 mL of Milli Q water through the sorbent at a slow drip rate. The 3 mL diluted loading fractions were then passed through their own respective MISPE at a slow flow rate of +-0.5 mL/min. Table 3.16 was used to prepare wash solvent fractions with an increasing methanol gradient. All 17 fractions, including the loading fraction, had a total volume of 3 mL and were passed through the sorbent at a slow drip-rate. As with the previous experiments, the more aqueous fractions passed through the sorbent with more difficulty compared to the fractions containing more methanol, the vacuum pressure was adjusted accordingly for each fraction to achieve the desired drip rate.

Table 3.16: Methanol: water gradient making up 17 fractions in a gradient from 0.8% to 80% methanol for chloroacetamide MISPE wash solvent optimization

Fraction #	Methanol (%)	Water (%)	Methanol (µL)	Water (µL)
1 (Load)	**0,83**	99,167	25,0	2975
2	**2**	98	60	2940
3	**4**	96	120	2880
4	**6**	94	180	2820
5	**8**	92	240	2760
6	**10**	90	300	2700
7	**12**	88	360	2640
8	**14**	86	420	2580
9	**16**	84	480	2520
10	**18**	82	540	2460
11	**20**	80	600	2400
12	**30**	70	900	2100
13	**40**	60	1200	1800
14	**50**	50	1500	1500
15	**60**	40	1800	1200
16	**70**	30	2100	900
17	**80**	20	2400	600

A 50 µL sample was taken from each 3 mL fraction and dried. The theoretical 100% elution efficiency was 16.67 µg/mL for each 3 mL fraction. Reconstitution was done with 50 µL methanol containing 5 µg/mL caffeine IS. The samples were then analysed by GC-MS utilizing the previously developed method in Section 3.2.

3.7.5: MTMIP methanol: water wash solvent gradient

From the NIP experiment (Section 3.7.2), it was found that a significant concentration of chloroacetamide pesticides started eluting after just 2% methanol. The aim of this experiment was to determine if there was a significant difference for the multi template (MT) MIP, compared to the NIP

and other MIPs in terms of wash solvent removing the template molecules. The loading fraction was prepared by adding a 25 µL methanol solution containing 2 mg/mL of each of the four pesticides to 9.9 mL water, to a total volume of 10 mL. The concentration for the loading fraction was thus 5 µg/mL.

The MTMISPE cartridge was conditioned by passing 3 mL of methanol, followed by 3 mL of Milli Q water through the sorbent at a slow drip rate. The 10 mL diluted loading fraction was then passed through the packed cartridge at a flow rate of +-0.5 mL/min. Table 3.17 displays all the fractions prepared that were passed through the MTMISPE cartridge at a slow drip rate and care was taken to never let the sorbent bed run dry.

Table 3.17: Methanol: water gradient making up 21 fractions in a gradient from 1% to 80% methanol for MTMIP wash solvent optimization

Fraction #	Methanol (%)	Water (%)	Methanol (µL)	Water (µL)
1 (Load)	**1**	99	100	9900
2	**2**	98	60	2940
3	**4**	96	120	2880
4	**6**	94	180	2820
5	**8**	92	240	2760
6	**10**	90	300	2700
7	**12**	88	360	2640
8	**14**	86	420	2580
9	**16**	84	480	2520
10	**18**	82	540	2460
11	**20**	80	600	2400
12	**22**	78	660	2340
13	**24**	76	720	2280
14	**26**	74	780	2220
15	**28**	72	840	2160
16	**30**	70	900	2100
17	**40**	60	1200	1800
18	**50**	50	1500	1500
19	**60**	40	1800	1200
20	**70**	30	2100	900
21	**80**	20	2400	600

A 50 µL sample was taken from each fraction in Table 3.17 and dried. The theoretical 100% elution efficiency was 16.67 µg/mL for each 3 mL fraction and 5 µg/mL for the 10 mL fraction. Subsequently 150 µL of the loading fraction was dried and the final theoretical concentration was calculated to be 15 µg/mL after reconstitution. Reconstitution was done with 50 µL methanol containing 5 µg/mL caffeine IS. The samples were then analysed by GC-MS utilizing the previously developed method in Section 3.2.

3.8: Recovery determination of spiked samples at relevant concentrations

This section describes how the packed MISPE cartridges were used for the extraction of pesticides from cannabis plant material.

3.8.1: Matrix matched calibration at lower concentrations for recovery determination

The calibration procedure was very similar to that described in Section 3.2.3; this however was a matrix matched calibration curve. The calibration standards were dried utilising vacuum concentration and then reconstituted with both a 25 µL solution containing 10 µg/mL caffeine IS and 25 µL of 20 mg/mL cannabis flower extract in methanol. The aim of the matrix matched calibration was to determine if there would be any significant difference between a calibration curve without matrix and the curve with matrix. Table 3.18 shows the concentrations used for each individual pesticide. Calibrations were performed separately for each of the four pesticides in triplicate statistical analysis.

The previous calibrations, from Section 3.2.3, were performed around 12.5 µg/mL, with two points above and two below this concentration. However, a lower calibration was desired so that less pre-concentration would be required; therefore a larger volume could thus be employed for reconstitution during sample preparation. Because the WS was dried, its volume was not accounted for in the final concentration calculation, only the IS solution and the methanol cannabis extract volumes were considered in this regard.

Table 3.18: Preparation guide for the matrix matched calibration curve for recovery determination of SPE, NISPE and MISPE

Calibration point	WS Volume (µL) (25 µg/mL pesticide in methanol)	Drying step and reconstitution	IS volume (µL) (10 µg/mL caffeine)	MeOH cannabis flower extract (µL) (20 mg/mL)	IS concentration (µg/mL)	Final pesticide concentration (µg/mL)
1	**1**	⇨	25	25	5	**0.5**
2	**2**		25	25	5	**1**
3	**5**		25	25	5	**2.5**
4	**8**		25	25	5	**4**
5	**10**		25	25	5	**5**

3.8.2: Extraction of pesticides from spiked cannabis samples

Although some variations in the plant material samples were evident, Figure 3.3 represents the most common type of sample, a bud or flower clipping. If the male and female plants are not separated by the grower, some of the buds can contain seeds. The buds may also vary in size depending on the strain, growing conditions and when the plant was harvested in its life cycle.

Figure 3.3: A typical cannabis plant sample of a bud clipping used to spike with pesticides for extraction purposes

The most difficult aspect of the analysis of these plant samples was found to be the volume of solvent needed to extract an analysable amount of pesticide, as the solvent and homogenised plant material form a slurry. Blending water and plant material together to form an aqueous slurry proved to be ineffective, as loading the aqueous slurry into the SPE cartridge proved problematic as the frits on the cartridge blocked or clogged. Filtering some of the aqueous solution from the slurry appeared to be ineffective at removing the triazine pesticides from the plant material, due to the low water solubility of the triazine pesticides (refer to Section 2.2.4). A more involved extraction procedure was found necessary, utilising an organic solvent to extract the pesticides more effectively. In a published study, extraction of 90 pesticides from homogenised and blended fruit and vegetable samples was achieved with acetone after which the extract was diluted with water prior to loading on the SPE cartridge (Štajnbaher & Zupančič-Kralj, 2003).

A mostly aqueous solution suitable for SPE is needed for the SPE loading fraction. It is well known (and used in QuEChERS), that acetonitrile can be separated from an acetonitrile/water solution by adding a salt, such as NaCl. This fact was found useful in the extraction of pesticides from the cannabis plant material.

3.8.2.1: Extraction procedure for cannabis plant material utilising acetonitrile and NaCl:

A bulk portion of cannabis plant material was blended into fine pieces using a blender. Approximately 3 g of fine plant material was weighed into a 50 mL PTFE centrifuge tube and spiked with pesticide standards to reach the MRL or limit. For a 3 g subsample, 0.15 µg of pesticide is required to reach the 0.05 mg/kg MRL, thus 6.0 µL of 25 ug/mL pesticide solution was used to spike the sample. Then 15 mL acetonitrile was added, and the sample was vortexed and sonicated (15 min). Enough water (20 mL)

to cover the sample was added to the plant material. The slurry was vortexed thoroughly and sonicated for 15 min. NaCl salt (500 mg) was added and the slurry vortexed thoroughly again. The sample was then centrifuged for 10 min at 3500 rpm. As much acetonitrile as possible was removed from the top acetonitrile layer utilising a pipette and transferred to a separate container. The acetonitrile fraction was evaporated to about 1 mL using a vacuum sample concentrator.

Just prior to SPE, the 1 mL concentrated acetonitrile sample was diluted with water. This dilution was dependent on both the SPE sorbent and on a target analyte, and was done ensure the dilution was sufficient for the target analytes to be trapped on the sorbent, as determined in Section 3.7. Although the experiments conducted in Section 3.7 were done with methanol and these dilutions were performed using acetonitrile, they were still found to be effective. This is because methanol has a polarity index value of 5.1, and acetonitrile is slightly more polar with a polarity index value of 5.8 (refer to Section 2.2.4). For example, if a 10% (v/v) acetonitrile solution was desired, 9 mL of water was added to 1 mL of acetonitrile.

Note: An emulsion is formed when the water is added to the acetonitrile, as the polarity of the solution moves to a polar solution from the less polar acetonitrile. Waxes and oils extracted from the plant into the acetonitrile can now no longer remain in solution. If this emulsion is not freshly prepared, the waxes and oils may separate completely from the water/ acetonitrile solution, and the pesticides may then also separate out of the solution with the other non-polar compounds. It was found useful to prepare the desired loading fraction ratio inside the SPE tube before turning on the vacuum on the SPE manifold.

3.8.2.2: Extraction procedure for cannabis plant material utilising repeated water washing

Each of the four selected pesticides are slightly soluble in water, although as shown in Table 2.5, the two triazine pesticides are less water-soluble at 33 and 9 μg/mL for atrazine and terbuthylazine, respectively. Acetochlor and alachlor are much more water soluble with over 200 μg/mL solubility for both pesticides.

A bulk portion of cannabis plant material was blended into fine pieces using a blender. Approximately 3 g of fine plant material was weighed out into a 50 mL PTFE centrifuge tube and spiked with pesticides to reach the desired MRL. For a 3 g subsample, 0.15 μg of pesticide is required to reach the 0.05 mg/kg MRL. Thus 6.0 μL of 25 ug/mL pesticide solution was used to spike the sample. Then 20 mL water was added and the slurry was vortexed thoroughly and sonicated for 15 min. The sample was then centrifuged for 10 min at 3500 rpm. As much water as possible was removed without disturbing the sediment and transferred to a separate container. Another 20 mL water was then added and the procedure was repeated.

The combined water fractions needed no further processing and was passed through the SPE cartridges as is. A double-sided needle with a rubber septum was found to be useful for passing the large quantity of water through the cartridge.

3.8.3: SPE methods used for recovery determination

The C18 SPE and MISPE methods were comparable to those described in Section 3.6, except they were performed on spiked cannabis samples.

3.8.3.1: C18 reference SPE method followed for the extraction of pesticides from cannabis extracts

The SPE cartridge was conditioned with 2 mL of methanol and then 2 mL of water. Before the SPE dried, the diluted extract prepared according to the procedures in Section 3.8.2 was loaded onto the cartridge at a slow drip rate, approximately 0.5 mL/min. A significant portion of pigment and other non-polar compounds extracted from the cannabis plant was observed to stick to the C18 sorbent. The SPE cartridge was then washed with 3 mL of 20% methanol: water at approximately 0.8 mL/min. Almost no pigment was removed during this washing step. The sorbent was dried under a vacuum of 50 kPa for 30 min. The pesticides were eluted using two 1.5 mL methanol fractions. The methanol fractions were added to the SPE cartridge while the manifold tap was closed to allow the sorbent to wet. The tap was then opened, and the elution was done under vacuum. The elution fractions were dried by vacuum concentration and they were then combined and reconstituted with 150 µL of 5 µg/mL caffeine in methanol solution. The theoretical concentration was thus 1 µg/mL for caffeine. The pesticide theoretical concentration was calculated for each separate sample. The samples were discernibly dirty at this stage, clearly not only pesticides were extracted from the plant. For the water extracts, only 50 µL of 5 µg/mL caffeine solution was required to redissolve the dried sample during reconstitution.

3.8.3.2: MISPE method followed for the extraction of pesticides from cannabis extracts

Section 3.5 describes how the MISPE and NISPE cartridges were packed. Prior to loading, the packed cartridge was conditioned with 2 mL methanol and then 2 mL of water. It is important to note the methanol fraction was analysed to ensure that no template molecule was eluting from the MISPE. Before the SPE dried, the diluted extract prepared according to the procedure in Section 3.8.2 was loaded onto the cartridge at a slow drip rate of approximately 0.5 mL/min. A significant portion of pigment and other non-polar compounds extracted from the cannabis plant was observed to pass through the synthesised sorbent and frit. The MISPE/NISPE cartridge was then washed with a previously determined % methanol fraction, depending on the MIP and target pesticide as determined in the wash solvent optimization sections (Section 3.3 and 4.6). All the wash and elution fractions used

are described in Table 3.19. Before the sorbent dried, the pesticides were eluted using two 1.5 mL methanol fractions. Elution was done under vacuum at a slow drip rate of approximately 0.5 mL/min. All the elution fractions were dried by vacuum concentration, combined and reconstituted with 150 µL of 5 µg/mL caffeine in methanol solution. The theoretical concentration was now 5 µg/mL for caffeine and for the pesticides each sample was specifically calculated based on the sample mass.

Note: It was observed that when a high vacuum was required (less than -15 mmHg) to pass the sample through the MISPE, the sorbent packing could be damaged and some of the sorbent pulled though the frit with the elution fraction. The sorbent could be effectively filtered out of the elution fraction using a 0.45 µm Sartorius syringe filter. The recovery was, however, always observed to be low when the sorbent was damaged, and the experiment was repeated. The high vacuum was only needed for the acetonitrile extracts and never for the water rinsing technique.

3.8.4: Summary of experiments performed for recovery determination of the template pesticides from the cannabis flower sample matrix.

Table 3.19 contains a summary of the C18 SPE experiments performed. Three replicates were performed for each pesticide utilising the two different extraction methods described in the subsections of Section 3.8.3. The pesticides were spiked and subsequently extracted simultaneously in their respective triazine and chloroacetamide classes. The amount of working standard (WS) was calculated for each sample based on its mass to reach the 0.05 mg/kg (or µg/g) concentration of the South African MRL on crops. The analyte specific loading, wash and elution fractions are also displayed in Table 3.19, as determined in Section 3.7. For loading larger fractions, a double-sided needle and rubber septum were found useful.

Table 3.19: Summary of the C18 SPE experiments performed for recovery determination of spiked cannabis plant material

Rep. (#)	Pesticide (s)	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS added (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
1	Atrazine & terbuthylazine	3145.4	0.05	**6.29**	Acetonitrile & NaCl	20 (6 mL)	20	70
2	Atrazine & terbuthylazine	3253.7	0.05	**6.51**	Acetonitrile & NaCl	20 (6 mL)	20	70
3	Atrazine & terbuthylazine	3059.1	0.05	**6.12**	Acetonitrile & NaCl	20 (6 mL)	20	70
4	Acetochlor & Alachlor	3023.1	0.05	**6.05**	Acetonitrile & NaCl	20 (6 mL)	40	70
5	Acetochlor & Alachlor	3000.9	0.05	**6.00**	Acetonitrile & NaCl	20 (6 mL)	40	70

Rep. (#)	Pesticide (s)	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS added (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
6	Acetochlor & Alachlor	3018.9	0.05	**6.04**	Acetonitrile & NaCl	20 (6 mL)	40	70
7	Atrazine & terbuthylazine	3082.5	0.05	**6.16**	Water rinsing	0 (+- 30 mL)	20	70
8	Atrazine & terbuthylazine	3188.6	0.05	**6.38**	Water rinsing	0 (+- 30 mL)	20	70
9	Atrazine & terbuthylazine	3242.6	0.05	**6.49**	Water rinsing	0 (+- 30 mL)	20	70
10	Acetochlor & Alachlor	2962.6	0.05	**5.93**	Water rinsing	0 (+- 30 mL)	40	70
11	Acetochlor & Alachlor	2940.9	0.05	**5.88**	Water rinsing	0 (+- 30 mL)	40	70
12	Acetochlor & Alachlor	3169.8	0.05	**6.34**	Water rinsing	0 (+- 30 mL)	40	70

The pesticides were spiked and subsequently extracted simultaneously in their respective triazine and chloroacetamide classes, as shown in Table 3.20. For the samples at a spiked concentration of 0.5 µg/g (replicates 13 to 24) much less sample was weighed (approximately 0.3 g) and half of the solvent described in Section 3.8.2 was used during the extraction procedure as so much less plant material was spiked.

Table 3.20: Summary of NISPE experiments performed for recovery determination from spiked cannabis plant material

Rep. (#)	Pesticide (s)	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
1	Atrazine & terbuthylazine	3017.2	0.05	**6.03**	Acetonitrile & NaCl	10 (6 mL)	10	70
2	Atrazine & terbuthylazine	2988.3	0.05	**5.98**	Acetonitrile & NaCl	10 (6 mL)	10	70
3	Atrazine & terbuthylazine	3032.7	0.05	**6.07**	Acetonitrile & NaCl	10 (6 mL)	10	70
4	Acetochlor & Alachlor	3015.6	0.05	**6.03**	Acetonitrile & NaCl	1 (15 mL)	1	70
5	Acetochlor & Alachlor	3180.8	0.05	**6.36**	Acetonitrile & NaCl	1 (15 mL)	1	70
6	Acetochlor & Alachlor	3019.5	0.05	**6.04**	Acetonitrile & NaCl	1 (15 mL)	1	70
7	Atrazine & terbuthylazine	3076.3	0.05	**6.15**	Water rinsing	0 (+- 30 mL)	10	70
8	Atrazine & terbuthylazine	2931.1	0.05	**5.86**	Water rinsing	0 (+- 30 mL)	10	70
9	Atrazine & terbuthylazine	3173.8	0.05	**6.35**	Water rinsing	0 (+- 30 mL)	10	70

Rep. (#)	Pesticide (s)	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
10	Acetochlor & Alachlor	2977.1	0.05	**5.95**	Water rinsing	0 (+- 30 mL)	1	70
11	Acetochlor & Alachlor	3036.8	0.05	**6.07**	Water rinsing	0 (+- 30 mL)	1	70
12	Acetochlor & Alachlor	2912.2	0.05	**5.82**	Water rinsing	0 (+- 30 mL)	1	70
13	Atrazine & terbuthylazine	297.8	0.5	**5.96**	Acetonitrile & NaCl	10 (3 mL)	10	70
14	Atrazine & terbuthylazine	311.8	0.5	**6.24**	Acetonitrile & NaCl	10 (3 mL)	10	70
15	Atrazine & terbuthylazine	315.5	0.5	**6.31**	Acetonitrile & NaCl	10 (3 mL)	10	70
16	Acetochlor & Alachlor	310.5	0.5	**6.21**	Acetonitrile & NaCl	1 (5 mL)	1	70
17	Acetochlor & Alachlor	305.7	0.5	**6.11**	Acetonitrile & NaCl	1 (5 mL)	1	70
18	Acetochlor & Alachlor	292.8	0.5	**5.86**	Acetonitrile & NaCl	1 (5 mL)	1	70
19	Atrazine & terbuthylazine	291.8	0.5	**5.84**	Water rinsing	0 (+- 15 mL)	10	70
20	Atrazine & terbuthylazine	305.6	0.5	**6.11**	Water rinsing	0 (+- 15 mL)	10	70
21	Atrazine & terbuthylazine	334.4	0.5	**6.69**	Water rinsing	0 (+- 15 mL)	10	70
22	Acetochlor & Alachlor	304.3	0.5	**6.09**	Water rinsing	0 (+- 15 mL)	1	70
23	Acetochlor & Alachlor	299.6	0.5	**5.99**	Water rinsing	0 (+- 15 mL)	1	70
24	Acetochlor & Alachlor	307.4	0.5	**6.15**	Water rinsing	0 (+- 15 mL)	1	70

Table 3.21 summarises the extraction experiments performed utilizing atrazine MISPEs to determine the recovery of atrazine and terbuthylazine on spiked cannabis flower samples. Atrazine and terbuthylazine were spiked together at the same concentration in each experiment. Each extraction procedure was repeated in triplicate for statistical analysis.

Table 3.21: Summary of atrazine MISPE experiments performed for recovery determination from spiked cannabis plant material

Rep. (#)	Pesticide	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
1	Atrazine & terbuthylazine	2991.5	0.05	**5.98**	Acetonitrile & NaCl	10 (6 mL)	10	70
2	Atrazine & terbuthylazine	2995.0	0.05	**5.99**	Acetonitrile & NaCl	10 (6 mL)	10	70

Rep. (#)	Pesticide	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
3	Atrazine & terbuthylazine	2953.7	0.05	**5.91**	Acetonitrile & NaCl	10 (6 mL)	10	70
4	Atrazine & terbuthylazine	2933.4	0.05	**5.98**	Water rinsing	0 (+- 30 mL)	10	70
5	Atrazine & terbuthylazine	2971.0	0.05	**5.94**	Water rinsing	0 (+- 30 mL)	10	70
6	Atrazine & terbuthylazine	3037.9	0.05	**6.08**	Water rinsing	0 (+- 30 mL)	10	70

Table 3.22 represents the experiments performed on the terbuthylazine MISPE to determine the recovery of atrazine and terbuthylazine spiked on cannabis flower samples. Atrazine and terbuthylazine were spiked simultaneously at the same concentration in each experiment. Each extraction procedure i.e. acetonitrile & NaCl and water rinsing, were repeated three times for statistical analysis purposes.

Table 3.22: Summary of terbuthylazine MISPE experiments performed for recovery determination of spiked cannabis flower samples

Rep. (#)	Pesticide	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
1	Atrazine & terbuthylazine	3024.7	0.05	**6.05**	Acetonitrile & NaCl	10 (6 mL)	10	70
2	Atrazine & terbuthylazine	2955.9	0.05	**5.91**	Acetonitrile & NaCl	10 (6 mL)	10	70
3	Atrazine & terbuthylazine	2930.7	0.05	**5.86**	Acetonitrile & NaCl	10 (6 mL)	10	70
4	Atrazine & terbuthylazine	2947.4	0.05	**5.89**	Water rinsing	0 (+- 30 mL)	10	70
5	Atrazine & terbuthylazine	3036.1	0.05	**6.07**	Water rinsing	0 (+- 30 mL)	10	70
6	Atrazine & terbuthylazine	2920.6	0.05	**5.84**	Water rinsing	0 (+- 30 mL)	10	70

Table 3.23 represents the extraction experiments performed utilizing acetochlor MISPEs to determine the recovery of the chloroacetamide pesticides on spiked cannabis flower samples. Acetochlor and alachlor were spiked together at the same concentration in each experiment. Each extraction procedure was repeated in triplicate for statistical analysis.

Table 3.23: Summary of acetochlor MISPE experiments performed for recovery determination from spiked cannabis plant material

Rep. (#)	Pesticide	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile	Wash % methanol	Elution % methanol
1	Acetochlor & alachlor	3082.1	0.05	**6.16**	Acetonitrile & NaCl	1 (15 mL)	1	70
2	Acetochlor & alachlor	2915.3	0.05	**5.83**	Acetonitrile & NaCl	1 (15 mL)	1	70
3	Acetochlor & alachlor	3063.8	0.05	**6.13**	Acetonitrile & NaCl	1 (15 mL)	1	70
4	Acetochlor & alachlor	2957.3	0.05	**5.91**	Water rinsing	0 (+- 30 mL)	0	70
5	Acetochlor & alachlor	3063.3	0.05	**6.13**	Water rinsing	0 (+- 30 mL)	0	70
6	Acetochlor & alachlor	3072.4	0.05	**6.14**	Water rinsing	0 (+- 30 mL)	0	70

Table 3.24 represents the experiments performed on the alachlor MISPE to determine the recovery of chloroacetamide pesticides on spiked cannabis flower samples. Acetochlor and alachlor were spiked simultaneously at the same concentration in each experiment. Each extraction procedure i.e. acetonitrile & NaCl and water rinsing, were repeated three times for statistical analysis purposes.

Table 3.24: Summary of alachlor MISPE experiments performed for recovery determination of spiked cannabis flower samples

Rep. (#)	Pesticide	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
1	Acetochlor & alachlor	2980.0	0.05	**5.96**	Acetonitrile & NaCl	1 (15 mL)	1	70
2	Acetochlor & alachlor	3033.2	0.05	**6.07**	Acetonitrile & NaCl	1 (15 mL)	1	70
3	Acetochlor & alachlor	2970.1	0.05	**5.94**	Acetonitrile & NaCl	1 (15 mL)	1	70
4	Acetochlor & alachlor	2916.1	0.05	**5.83**	Water rinsing	0 (+- 30 mL)	0	70
5	Acetochlor & alachlor	2927.5	0.05	**5.86**	Water rinsing	0 (+- 30 mL)	0	70
6	Acetochlor & alachlor	2964.0	0.05	**5.93**	Water rinsing	0 (+- 30 mL)	0	70

Table 3.25 represents the experiments performed on the multi-template MISPE to determine the recovery of all four imprinted polymers on spiked cannabis flower samples. Atrazine, terbuthylazine acetochlor and alachlor were spiked simultaneously at the same concentration in each experiment. Each extraction procedure i.e. acetonitrile & NaCl and water rinsing, were repeated three times for statistical analysis purposes.

Table 3.25: Summary of MT MISPE experiments performed for recovery determination of spiked cannabis flower samples

Rep. (#)	Pesticide	Flower sample mass (mg)	Spiked concentration (µg/g)	25 µg/mL WS to add (µL)	Extraction method	Loading % acetonitrile (volume)	Wash % methanol	Elution % methanol
1	Atrazine, terbuthylazine acetochlor & alachlor	3031.2	0.05	**6.06**	Acetonitrile & NaCl	1 (15 mL)	1	70
2	Atrazine, terbuthylazine acetochlor & alachlor	3027.1	0.05	**6.05**	Acetonitrile & NaCl	1 (15 mL)	1	70
3	Atrazine, terbuthylazine acetochlor & alachlor	2920.4	0.05	**5.84**	Acetonitrile & NaCl	1 (15 mL)	1	70
4	Atrazine, terbuthylazine acetochlor & alachlor	3076.6	0.05	**6.15**	Water rinsing	0 (+- 30 mL)	0	70
5	Atrazine, terbuthylazine acetochlor & alachlor	3051.2	0.05	**6.10**	Water rinsing	0 (+- 30 mL)	0	70
6	Atrazine, terbuthylazine acetochlor & alachlor	2900.7	0.05	**5.80**	Water rinsing	0 (+- 30 mL)	0	70

3.9: Statistical analysis

3.9.1: Paired t-test

The paired t-test is used to compare two data sets, typically generated from two different methods. The t-test is used to determine if there is a statistical difference between the two sample sets, the following equation is used:

$$t = \frac{m_A - m_B}{\sqrt{\frac{S^2}{n_A} + \frac{S^2}{n_B}}}$$

To test the null hypothesis, we test whether mean of the first sample set (m_A) differs significantly from mean of the second sample set (m_B) while considering the standard deviation and number of data points in the equation for statistic *t. S* represents the standard deviation of each sample set while n_A and n_B represents the number of replicates of each sample set. When selecting a t_{crit} value to test the hypothesis against, the degrees of freedom and significance level must be considered. A small p-

value (typically ≤ 0.05) indicates strong evidence against the null hypothesis; therefore, the null hypothesis is rejected. The degrees of freedom is required to determine the t_{crit} value to test against the above formula, where the degrees of freedom is calculated as follows: $n_A + n_B - 2$ (Miller & Miller 2010-Chapter 3.4).

3.9.2: Limit of quantitation and detection

A sufficient concentration of analyte must be present in a sample to produce a detectable signal and distinguish the analyte from the noise or background of the sample matrix during analysis. The detection limit (LOD), is taken as the lowest concentration of an analyte in a sample that can be detected, although it might be unquantifiable, with the standard analysis conditions. The limit of quantitation (LOQ) is defined as the lowest concentration an analyte can be quantified with acceptable accuracy and precision. Conventionally a signal to noise ratio of three is considered acceptable for the LOD, and a signal to noise ratio of 10 is necessary for quantitation.

For a linear calibration curve, linear regression can be used to estimate the LOQ. For a linear calibration curve, the following formula applies:

$$y = mx + c$$

Where *y* is the instrument response, or the response ratio of the analyte to internal standard, *m* is the slope of the line, *x* represents the analyte concentration and *c* is the y intercept of the curve. For a curve forced through zero, *c* = 0. The linear range of an analytical method is where *y* is linearly related to the concentration, *x.* The limit of quantitation is then calculated utilising the equation:

$$LOQ = 10S_y/m$$

Where S_y is the standard deviation of the curve or response (*y* value) and *m* is the slope of the line.

There exists a function in Excel to calculate the standard error of the calculated y response compared with the known z value. The Excel function has the following format: "=STEYX(*calculated _ys*, *known_xs*)" where the range y values are placed in the first part of the function and x values are placed in the second part of the function. The standard error is a measure of the degree of deviation in the prediction of y values for an individual x value.

3.9.3: Relative standard deviation (RSD)

The precision of an analytical method can be defined as the random or indeterminate error associated with a measurement. Statistically, this error can be represented by the standard deviation or relative standard deviation (RSD). RSD is very useful to compare the precision of different analytical methods (Fifield & Kealey, 1991). Standard deviation is calculated as follows:

$$S = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

Where *n* represents the number of data points, $\bar{x}$ represents the mean and x_i each of the individual data points in the sum calculation.

The following formula is used to calculate %RSD:

$$RSD\% = \frac{s}{\bar{x}} \times 100$$

Where *s* represents standard deviation and $\bar{x}$ is the average of the analysis results (Fifield & Kealey, 1991).

Chapter 4 : Results and discussion

The first section of this chapter presents the NIP and MIP synthesis results, followed by the results relating to the GC-MS method which include calibration curves and example chromatograms. Section 4.3 contains the results and discussion for the adsorption capacity experiments, whilst the characterization of the synthetic polymers is discussed in Section 4.4 followed by SPE and wash solvent optimization. Finally, the MISPE results for the extraction of the selected pesticides from cannabis samples is discussed in Section 4.7.

4.1: NIP and MIP synthesis results

The following sections will discuss the amounts and yields obtained for both MIP and NIP synthesis procedures as described in Section 3.1.

4.1.1: NIP synthesis results

The dry product masses were as follows: reaction 1 = 115.2 mg, reaction 2 = 113.7 mg and reaction 3 = 120.5 mg for the three small scale NIP syntheses (Figure 4.1) employing differing amounts of initiator.

Figure 4.1: The three NIP reaction products after 24h incubation time in the oven

After the NIPs as seen Figure 4.1 were dried, all three NIPs were fine powders of a bright white colour.

The theoretical mass of the dry NIPs can be calculated by adding the masses of the monomers and initiator reagents. The molecular mass for AIBN is 164.21 g/mol, thus if 0.03 mmol AIBN was used, a mass of 4.92 mg AIBN was added to the reaction mixture. Table 4.1 contains all the masses and calculated yields for the three NIP reactions.

Table 4.1: Yield calculations for NIP synthesis based on the dried mass of the polymer

Reaction	MAA (mg)	EDGMA (mg)	AIBN (mg)	Total theoretical mass (mg)	Measured mass (mg)	Yield (%)
1	34.4	79.2	4.92	118.52	115.2	97.2
2	34.4	79.2	9.85	123.45	113.7	92.1
3	34.4	79.2	14.78	128.38	120.5	93.9

It can thus be concluded that polymerization reactions can take place over a range of AIBN concentrations and without the removal of the MEHQ inhibitor. The yields were all slightly below 100%, as it was possible that some of the NIP was lost during washing, drying and transfer to the weighing boat. Some unreacted monomer or crosslinker could also have been removed by the washing steps.

4.1.2: Small scale MIP synthesis results

Table 4.2 shows the calculation of the yields of the small-scale MIP synthesis procedure. The GC vials were pre-weighed to minimize the transfer of the synthesised MIPs to different vials and their subsequent loss. The observed yields were found to be higher than for the NIPs. All four MIPs were white in colour with a fine powder consistency.

Table 4.2: The MIP mass before template removal and associated yields

Vial	Template	Empty vial (mg)	Vial + MIP+ template (mg)	MIP + template mass (mg)	Theoretical mass (mg)	Yield (%)
1	Atrazine	2354.1	2477.6	123.5	126.4	97.7
2	Terbuthylazine	2353.0	2476.0	123.0	126.7	97.1
3	Acetochlor	2351.2	2475.4	124.2	127.7	97.3
4	Alachlor	2351.1	2475.7	124.6	127.7	97.6

From Table 4.2, the yields appear to be similar to one another and the NIP synthesis procedure, thus the templates do not significantly affect the free radical polymerization reaction in terms of polymerization efficiency. There was a very large atrazine peak found upon analysis of the supernatant methanol solution from the atrazine MIP after the Soxhlet extraction was run for 36 h indicating that the extraction process was effective. Unfortunately, during the drying process, the atrazine MIP was lost, as the fine powder was dispersed by the fan in the drying oven. Thus in future experiments other drying methods or sample containers in the oven were used.

4.1.3: Batch scale MIP synthesis results

The final mass of NIP obtained was 609.0 mg, and Table 4.3 shows the polymer masses for all the synthesis replicates. The average yield was calculated based on the expected mass compared to the measured mass as follows:

$$\%Yield = \frac{Polymer\ mass}{EDGMA + MAA + AIBN + Template} \times 100$$

$$\%Yield = \frac{Polymer\ mass}{396\ mg + 172\ mg + 37.6\ mg + Template} \times 100$$

$$\%Yield = \frac{Polymer\ mass}{605.6\ mg + Template} \times 100$$

All the yields were larger than 100%, meaning a little toluene remained in the MIP. Although further drying did not appear to affect this, perhaps some toluene was also trapped in the polymer or a mass error was made, in most cases there was a small enough difference to be explained by random error, such as pipetting error.

The MIP and atrazine had a combined mass of 613,4 mg for example, and this is roughly equivalent to the mass of the MMA, EGDMA, AINB and atrazine added to the reaction. It could thus be concluded the toluene had been fully dried.

Table 4.3: Summary of the mass and average yields of the replicate MIP bulk batch synthesis

	MIP Type	Repeat 1 (mg)	Repeat 2 (mg)	Repeat 3 (mg)	Repeat 4 (mg)	Total mass (mg)	Average yield (%)
1	Atrazine	613.4	614.3	612.2	616.8	2456.7	100.7
2	Terbuthylazine	614.7	616.0	620.2	620.2	2471.1	101.3
3	Acetochlor	612.7	617.5	617.4	616.6	2464.2	100.8
4	Alachlor	617.2	620.1	613.4	618.4	2469.1	101.1
5	NIP	609.0	607.4	608.3	611.2	2435.9	100.8

There did not appear to be any significant difference between the repeats or between the syntheses with different templates.

After the synthesis, the template was removed by washing the MIP seven times with 9:1 methanol: glacial acetic acid solution. A sample was taken from each wash fraction. The total mass of the atrazine removed from the MIP was then determined using the calibration curve. The combined wash fractions contained approximately 152.7 µg/mL atrazine. There was 23 mL of wash fraction in total, meaning a total of 3512.1 µg atrazine had been removed from the MIP. Considering the total atrazine used in the synthesis was 5.4 mg, there was still approximately 1887.9 µg atrazine potentially trapped in the MIP.

Further extraction was thus required or perhaps the atrazine was completely enclosed in the polymer and was thus inaccessible to the solvent. After the seven wash cycles, no more atrazine could be detected in the solution obtained from a further wash step.

4.1.4: Enhanced adsorption capacity MIP synthesis results

Figure 4.2 shows the products of a typical bulk polymerization reaction after the vial was removed from the oven. There is a clear layer of toluene over the polymer, and after the polymer had been dried it became bright white, as observed in Figure 4.2.

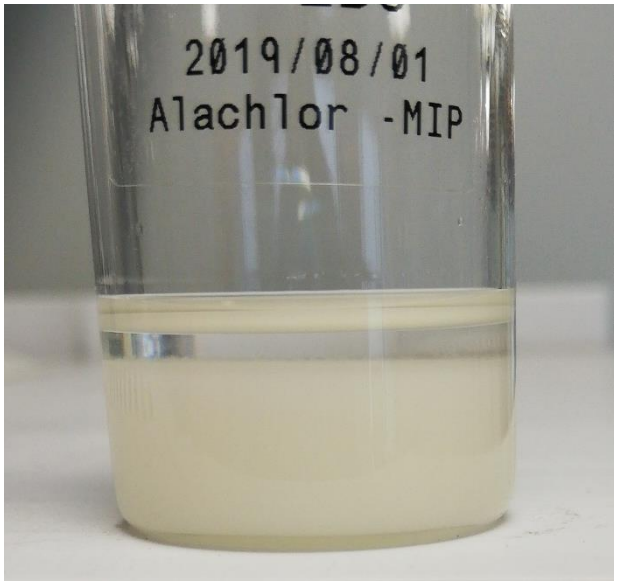


Figure 4.2: An example of the batch scale alachlor MIP after completion of the 24h 60 °C incubation period

The yields of each EAC MIP were calculated in the same way as described in the previous MIP synthesis sections. The yields for the dried MIPs before and after template removal as well as the theoretical mass, calculated from the masses of the reagents, are shown in Table 4.4.

Table 4.4: EAC MIP synthesis yields for each separate template reaction before and after template removal

EAC MIP Class	Dried MIP- before template removal (mg)			Post template removal (mg)			Average yield prior to template removal (%)	Average yield after template removal (%)
	Rep 1	Rep 2	Theoretical mass	Rep 1	Rep 2	Theoretical mass		
Atrazine	635.1	629.0	627.1	555.9	574.3	605.5	100.79	93.32
Terbuthylazine	666.8	635.3	628.5	575.7	564.3	605.5	103.59	94.13
Acetochlor	628.7	641.3	632.5	546.3	573.6	605.5	100.39	92.47
Alachlor	653.8	639.7	632.5	567.7	567.8	605.5	102.25	93.76

All the yields before template removal are > 100%. This might indicate the EAC MIP was not 100% dried, or there are small errors in pipetting and weighing resulting in the determined yield. The mass lost after template removal is greater than expected, this indicates some of the EAC MIP was lost during washing and transfer steps.

4.1.5: Multi template MIP synthesis results

The theoretical total mass was calculated to be 654.5 mg and the yield was calculated with the following equation for the multi template molecularly imprinted polymer (MTMIPs):

$$\%Yeild = \frac{Polymer\ mass}{EDGMA + MAA + AIBN} \times 100$$

Two batches of the MTMIP were prepared, and the final dried mass after the template was removed is reported in Table 4.5 with the calculated yield.

Table 4.5: Yields for multi-template MIP synthesis after the templates were removed

Batch #	Dried mass after template removal (mg)	% yield
1	603.2	92.2
2	598.2	91.4
Average	600.7	91.8

On average, 53.8 mg of the total mass was lost during template removal. The total template mass only accounts for 49 mg. This means a small amount of polymer was lost during the washing process, as can be expected.

4.1.6: Discussion of the template removal process

After the MIP synthesis as described in Section 3.1, several attempts were made to remove the template molecules from the MIP. Firstly, a Soxhlet extraction was performed on the synthesised MIP containing the atrazine template. The Soxhlet was run for over 2 days, roughly for 36 hours. Methanol was used as solvent and the temperature was set to 65 °C as described in Section 3.1.2. The Soxhlet was effective, but very time and energy consuming, and since many replicates and variations of MIPs were synthesised with the four different templates, a faster method that could be used to simultaneously remove the template from several different MIPs was required. The rinsing method described in Section 3.1.3 was thus attempted, where approximately 1 g of MIP was weighed into a 15 mL PTFE centrifuge tube, and a 5 mL solution of 9:1 methanol: acetic acid was added. The centrifuge tube was vortexed to ensure all the polymer was suspended in solution and it was then centrifuged at 3500 rpm for 5 min. The supernatant was decanted carefully, as to disturb the sedimented MIP as little as possible. This procedure was repeated seven times in the same centrifuge tube. Although the process is more labour intensive, the MIPs could be washed much more quickly and a set of 8 tubes can be placed into the centrifuge at once, allowing for the template removal of several MIPs in a few hours.

An observation was made that when the MIPs were ground with a mortar and pestle during the size fractionation procedure, and after the freshly ground and sieved MIPs were submerged in methanol, pesticide template was detected in the methanol where before this process no extracted template was detected. Additional template was thus released by the grinding process. It can be hypothesised that template sites that were completely enclosed by the polymer, were exposed by this process (which could also occur at low pHs). It was thus found necessary to remove more template after the sieving process. If the MIP was packed into a MISPE, and a 9:1 methanol: acetic acid solution was passed through the freshly packed cartridge some template was detected in the eluate. If enough eluent is passed through the MISPE, all the template was found to be removed successfully after about 20 mL washing solution had passed through the sorbent bed.

4.2: Optimization of the GC-MS method

Section 4.2 discuses all optimization aspects of the GC-MS method with regards to chromatograms, target analyte peak shapes and calibration curves.

4.2.1: Initial GC-MS method development

To verify retention times of the analytes of interest the GC-MS analysis was first run in scan mode as discussed in Section 3.2. Figure 4.3 displays a scan chromatogram indicating the target analytes and their respective retention times. A relatively lower response was observed for caffeine although the concentrations were equivalent. The ion fragments of caffeine thus have a lower summed intensity compared to the other analytes for the ions monitored in this total ion current (TIC), although it should be noted that some intensity was lost since ions with an *m/z* < 50 were omitted. In terms of the GC method, there was clear baseline separation for all the components for a relatively short runtime as all the analytes were eluted before 5 min. The peak shape was observed to be symmetrical without fronting or tailing for all analytes. Since a splitless 1 µL injection was performed and the concentration was known to be 12.5 µg/mL, the on-column amount was calculated to be 12.5 ng for all the analytes.

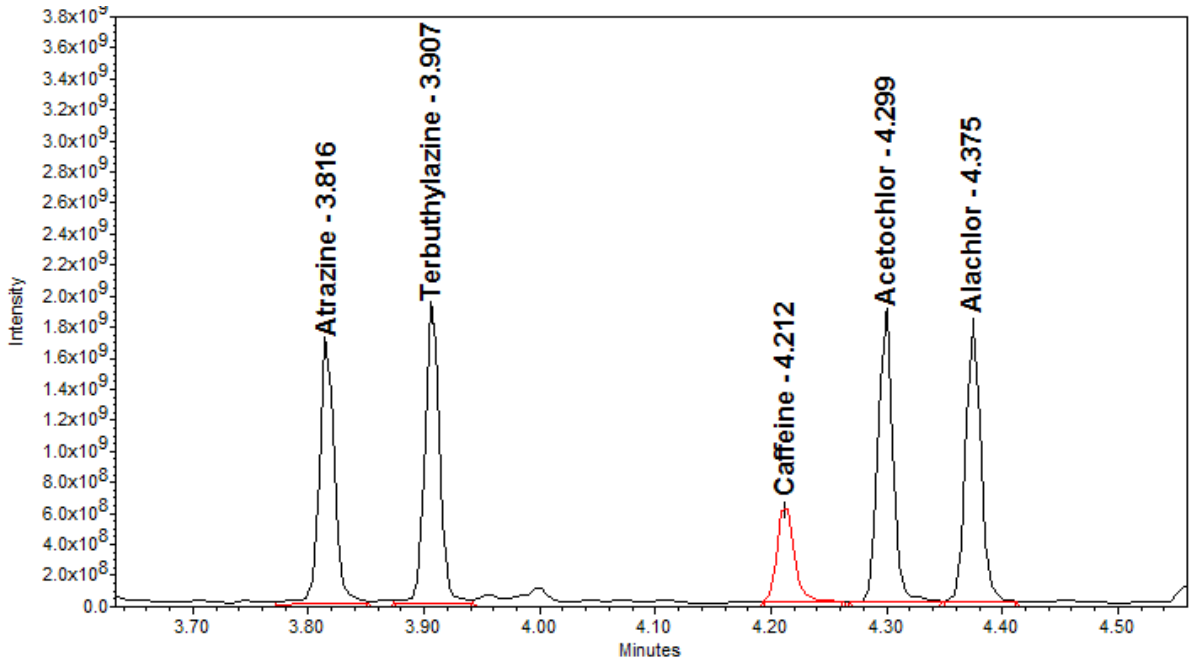


Figure 4.3: TIC chromatogram obtained from the GC-MS in scan mode (m/z of 50 to 300 Da) of a 12.5 µg/mL standard at 1 µL injection volume for all the components including caffeine as internal standard

Table 4.6 shows the peak retention times and the NIST library matches for each target analyte in the mixed standard. The ion ratios in a mass spectrum depend largely on the MS tune file and instrumental parameters and a perfect match to the library spectra is thus rare.

Table 4.6: The scan GC-MS chromatogram NIST search results and MS spectrum library matches expressed in %

Peak retention time (min)	Highest NIST Library match	% Match
3.814	Atrazine	92.6
3.905	Terbuthylazine	90.7
4.210	Caffeine	92.2
4.300	Acetochlor	94.3
4.375	Alachlor	90.7

With the retention times now confirmed, a single ion recording (SIR) method was set up as detailed in Section 3.2. Figure 4.4 contains the overlaid chromatograms for each of the main quantitation ions for all the analytes of interest. It should be noted that some SIR chromatograms contain more than one peak. The acetochlor chromatogram has quite a high secondary peak at 4.371 min, this represents the mass fragment of alachlor at *m/z* 146, eluting at 4.37 min. The monitored *m/z* representing the caffeine mass fragment at 194 Da, has two additional peaks at 4.293 and 4.370 min, representing the smaller mass fragments of alachlor and acetochlor, respectively. Refer to Section 2.6 and the appendices for fragmentation patterns and structures of each pesticide.

Table 3.7 contains a summary of the quantitation and qualifier ions for each analyte. For the first window (3.5 – 4.9 min), the dark blue chromatogram represents a *m/z* of 200 Da and is the main quantitation mass fragment for atrazine. The light blue chromatogram represents a *m/z* 214, the quantitation fragment of terbuthylazine. Green represents a *m/z* of 215 and black a *m/z* of 173. The pink and red chromatograms represent *m/z* 43 and 58 respectively, which are also major mass fragments of atrazine and terbuthylazine but were not selected as quantitation ions due to concerns regarding potential interferences in the lower mass range. In the second SIR window (4.8 – 6 min) the red chromatogram represents a *m/z* of 45 and is the mass fragment with the highest response for alachlor. There are several shades of blue chromatograms with a peak at 4.3 min representing mass fragments of acetochlor with *m/z* values of 59, 146 and 162. Green and pink represents *m/z* values of 194 and 109 respectively, which are the mass fragments for caffeine used as internal standard for quantitation purposes.

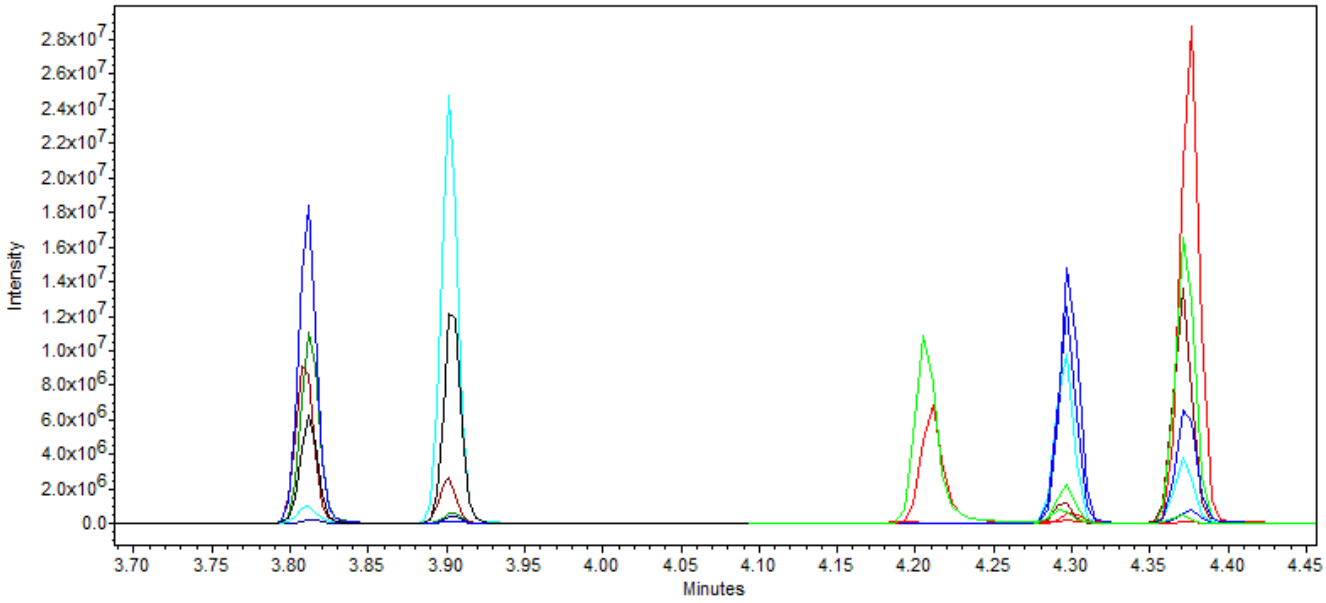
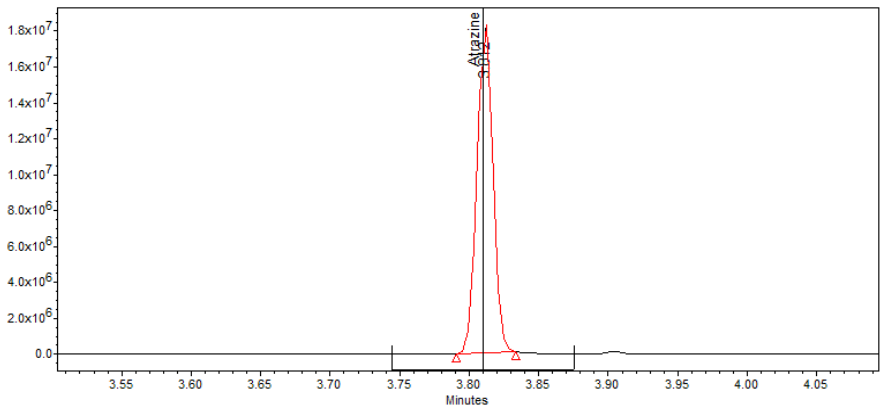
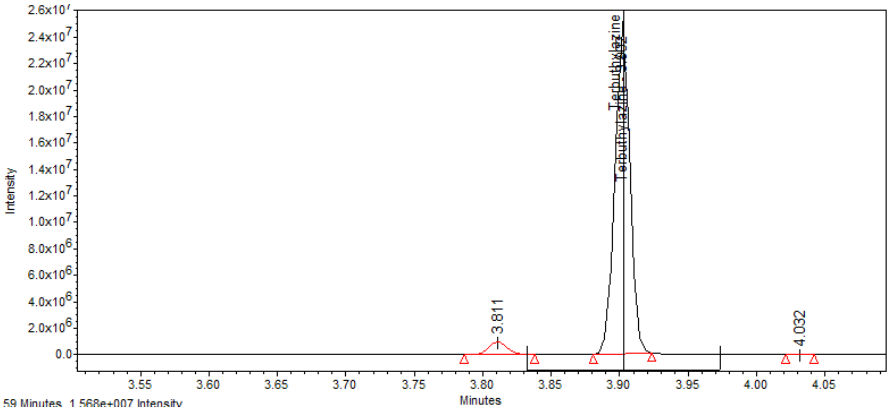
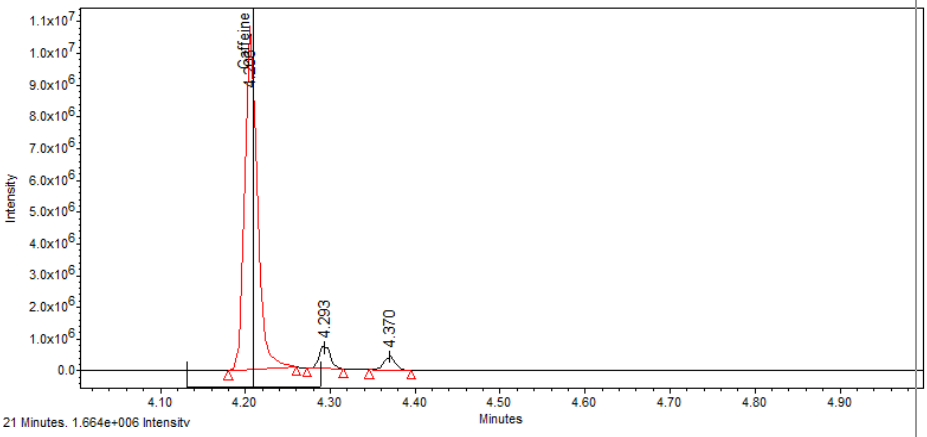
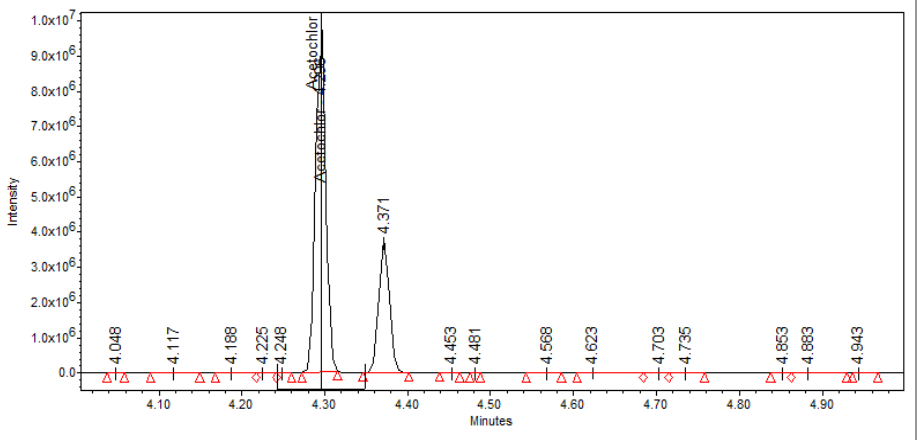


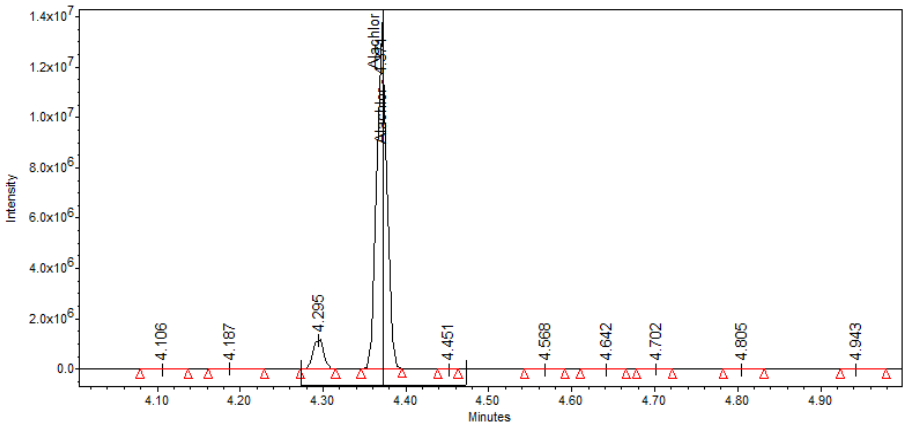
Figure 4.4: Overlaid SIR chromatograms, in different colours, at 12.5 µg/mL (12.5 ng on column) for all the monitored compounds in a single 1 µL injection. The elution order and retention times of the analytes are identical to the TIC in Figure 4.3

The software allows for the selection of each SIR chromatogram separately, as seen in Table 4.7, for the quantitation mass fragments of each analyte. Each chromatogram has a highlighted retention time window for each analyte.

Table 4.7: Individual SIR chromatograms that shows the quantitative mass spectrum for each analyte from a standard solution at 12.5 µg/mL (12.5 ng on column)

Peak	*m/z*	Chromatogram
Atrazine	200	

Peak	*m/z*	Chromatogram
Terbuthylazine	214	
Caffeine	194	
Acetochlor	146	

Peak	*m/z*	Chromatogram
Alachlor	160	

4.2.2: Calibration curves

This section presents the results obtained for the first calibrations performed during GC-MS method development. These calibration curves were also later used for statistical validation of the quantitation method. A five-point calibration curve was performed for each pesticide and four replicates were performed (n=4) at each concentration point (Table 4.8). The expected concentration for each point is displayed along with the experimentally determined value. The % deviation from the expected value was then calculated for every data point on the calibration curve.

Table 4.8: Summary of the 20 calibration points for the calibration of target pesticides

Point #	Expected concentration (µg/mL)	Alachlor		Acetochlor		Terbuthylazine		Atrazine	
		Calc. conc. (µg/mL)	% Deviation	Calc. conc. (µg/mL)	% Deviation	Calc. conc. (µg/mL)	% Deviation	Calc. conc. (µg/mL)	% Deviation
1	2.5	2.51	0.55	2.53	1.16	2.48	-0.94	2.51	0.33
2	2.5	2.45	-1.88	2.47	-1.09	2.42	-3.35	2.40	-4.19
3	2.5	2.49	-0.33	2.51	0.57	2.57	2.77	2.57	2.95
4	2.5	2.50	-0.09	2.52	0.70	2.63	5.33	2.67	6.82
5	5	5.58	11.64	5.55	11.08	5.61	12.14	5.53	10.56
6	5	4.58	-8.36	4.58	-8.40	4.66	-6.81	4.66	-6.86
7	5	4.53	-9.38	4.51	-9.70	4.44	-11.15	4.33	-13.30
8	5	4.60	-7.94	4.61	-7.86	4.73	-5.47	4.80	-4.04
9	10	10.09	0.87	9.98	-0.20	9.98	-0.22	9.96	-0.45
10	10	10.69	6.88	10.64	6.38	10.36	3.57	10.38	3.80
11	10	10.37	3.67	10.30	2.97	9.90	-1.03	9.61	-3.86
12	10	10.05	0.46	9.99	-0.07	10.18	1.74	10.32	3.20
13	15	16.08	7.18	15.78	5.20	16.13	7.54	15.98	6.51
14	15	15.81	5.39	15.73	4.80	15.36	2.38	15.52	3.50
15	15	14.48	-3.46	14.55	-2.98	14.68	-2.12	14.81	-1.26

Point #	Expected concentration (µg/mL)	Alachlor		Acetochlor		Terbuthylazine		Atrazine	
		Calc. conc. (µg/mL)	% Deviation	Calc. conc. (µg/mL)	% Deviation	Calc. conc. (µg/mL)	% Deviation	Calc. conc. (µg/mL)	% Deviation
16	15	15.49	3.28	15.41	2.75	15.69	4.60	15.92	6.10
17	20	20.53	2.66	20.70	3.48	20.63	3.17	20.32	1.61
18	20	18.92	-5.39	19.09	-4.57	19.32	-3.42	19.34	-3.29
19	20	18.63	-6.84	18.64	-6.81	18.30	-8.49	18.37	-8.15
20	20	19.41	-2.97	19.72	-1.41	19.95	-0.24	20.00	0.01

As seen in Table 4.8, all the variances in the calibration points were below 15%. From these 20 data points, a calibration curve was drawn up for each of the four pesticides. Table 4.9 contains the line equation for each calibration curve and the coefficient of determination (R^2). All the R^2 values are close to 1, thus indicating a strong linearity between the area ratio and the concentration.

Table 4.9: The internal standard corrected calibration curve formulas for the pesticides of interest

Pesticide	R^2	Line equation
Alachlor	0.9982	Y = 2.002e-001 X + 1.21e-002
Acetochlor	0.9980	Y = 1.978e-001 X + 2.25e-002
Terbuthylazine	0.9978	Y = 1.984e-001 X + 1.67e-002
Atrazine	0.9992	Y = 1.963e-001 X + 1.88e-002

The mean of the four replicates at each concentration was calculated for each of the four pesticides. The standard deviation (STDEV) was also calculated for each of the four replicates as shown in Table 4.10.

Table 4.10: The mean and standard deviation for the pesticide calibration curves

Expected concentration (µg/mL)	Alachlor (µg/mL)		Acetochlor (µg/mL)		Terbuthylazine (µg/mL)		Atrazine (µg/mL)	
	Mean	STDEV	Mean	STDEV	Mean	STDEV	Mean	STDEV
2.5	2.48	0.02	2.50	0.02	2.52	0.10	2.53	0.12
5	4.87	0.60	4.86	0.60	4.85	0.51	4.82	0.50
10	10.29	0.29	10.22	0.31	10.10	0.21	10.07	0.36
15	15.46	0.69	15.36	0.56	15.46	0.61	15.55	0.53
20	19.37	0.84	19.53	0.89	19.55	0.99	19.51	0.86

From Table 4.10 new calibration curves were drawn up with the area ratio (analyte peak area/ IS peak area) on the y axis and the expected concentration on the x axis. The standard deviation is displayed by error bars for each calibration point. All four calibration curves are displayed on separate axes with their R^2 values and formulas displayed, in Figure 4.5.

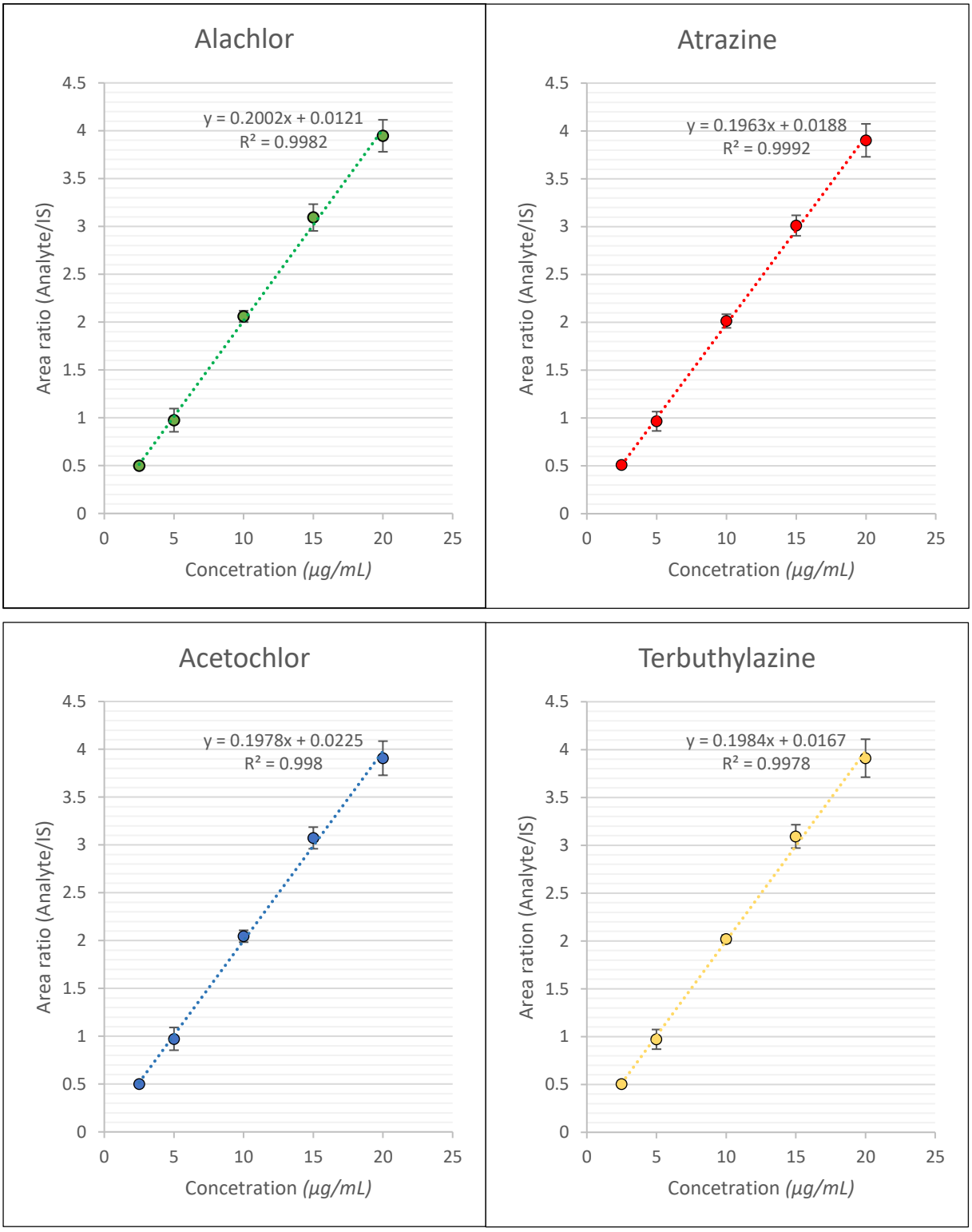


Figure 4.5: Calibration curves of all four analytes of interest with error bars displaying the calculated standard deviations (n=4 for each concentration point)

4.2.3: Statistical analysis and validation

Correlation coefficient values were high with R^2 >0.99 for the calibration curves as seen in Table 4.9 and Figure 4.5. The use of caffeine as an internal standard enabled the calibration curve to stay valid for a longer period of time because area ratio is monitored instead of just signal intensity. The use of internal standards counters the loss of signal intensity as the MS electron ionization source gets dirty over time. During the study, regular control samples were analysed to evaluate the accuracy of the calibration curve and to determine whether recalibration was required. Since the instrument was not only used for this study, matrix effects and contamination could also cause the calibration curve to become invalid. If the control samples were found to have a bias from the calibration curve, a recalibration was performed, in the same manner as described in Section 3.2.3.

Calculated LOQ

The limit of quantitation (LOQ) was calculated using the formulae in Section 3.9.2, where the calibration curve standard deviation and slope are used. The slope of the calibration curve and the standard deviation were used as displayed in the calibration curve formulas as per Table 4.9 and the statistical analysis of Table 4.10. Table 4.11 lists the LOQ for the relevant pesticides.

Table 4.11: LOQ as calculated from the calibration curve slope and standard deviation

Pesticide	LOQ (µg/mL)
Atrazine	1.16
Terbuthylazine	0.97
Acetochlor	0.25
Alachlor	0.26

It is interesting to note that atrazine and terbuthylazine had a significantly higher LOQ than acetochlor and alachlor. This is due to the higher standard deviation of the atrazine and terbuthylazine calibration curves.

E**xperimentally determined LOD and LOQ**

As explained in Section 3.9.2, the signal to noise ratio (S/N) can also be used to experimentally determine the LOD and LOQ. A series of lower concentration standard injections, from 0.5 to 0.05 µg/mL, were performed to determine the S/N for each analyte and the subsequent approximate LOD and LOQ values.

The chromatograms in Figure 4.6 were obtained for a 0.5 µg/mL injection of each pesticide. The chromatograms were left unsmoothed so the noise could be clearly observed in each case. Figure 4.6 shows the chromatogram for atrazine at 0.5 µg/mL, with a peak height of 4487.6 and a noise of around

500, this was determined to be just below the LOQ for atrazine at a S/N value of 9.3, thus approximately 10. At 1 µL injection volume, this is 0.5 ng on column injection. The peak shape was found not to be ideal at this low concentration level.

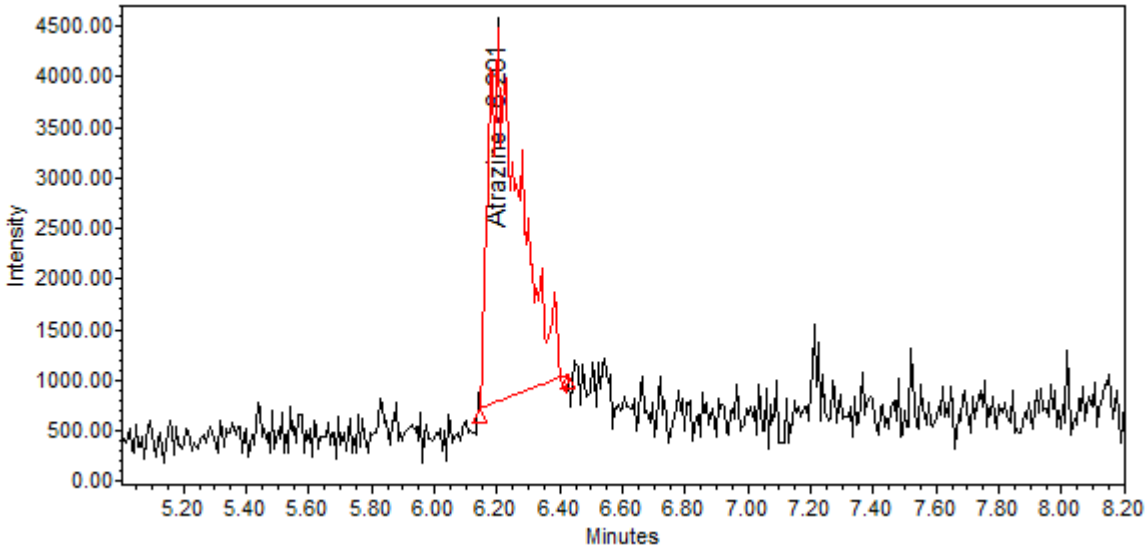


Figure 4.6: Atrazine (m/z 200) SIR chromatogram for a 0.5 µg/mL atrazine solution with a 1 µL injection volume (0.5 ng on column)

Figure 4.7 displays the terbuthylazine SIR chromatogram obtained at a concentration of 0.5 µg/mL. This was found to be above the LOQ for terbuthylazine as the S/N was found to be 26. Considering the 1 µL injection volume, this means 0.5 ng was injected on column. The peak shape was found to be far sharper for terbuthylazine at this low concentration compared to atrazine.

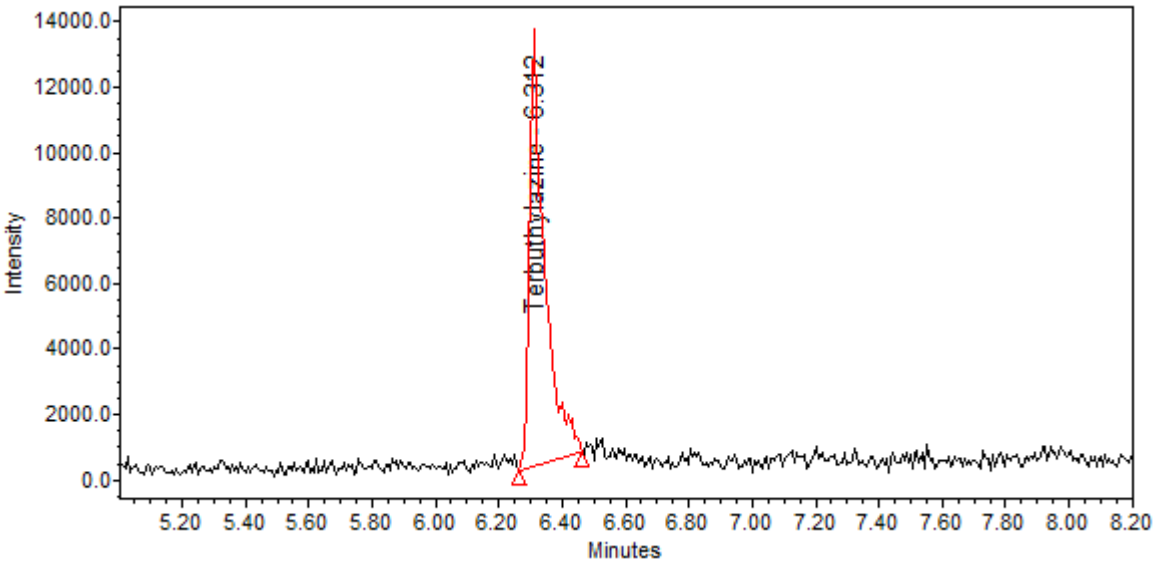


Figure 4.7: SIR chromatogram of terbuthylazine (m/z 214) at 0.5 µg/mL with a 1 µL injection volume (0.5 ng injected on column)

Figure 4.8 and 4.9 represent chromatograms for acetochlor and alachlor respectively, at 0.5 µg/mL. Both the acetochlor and alachlor peaks were found to be well above their LOQs with S/N values of 21.8 and 16.6 respectively.

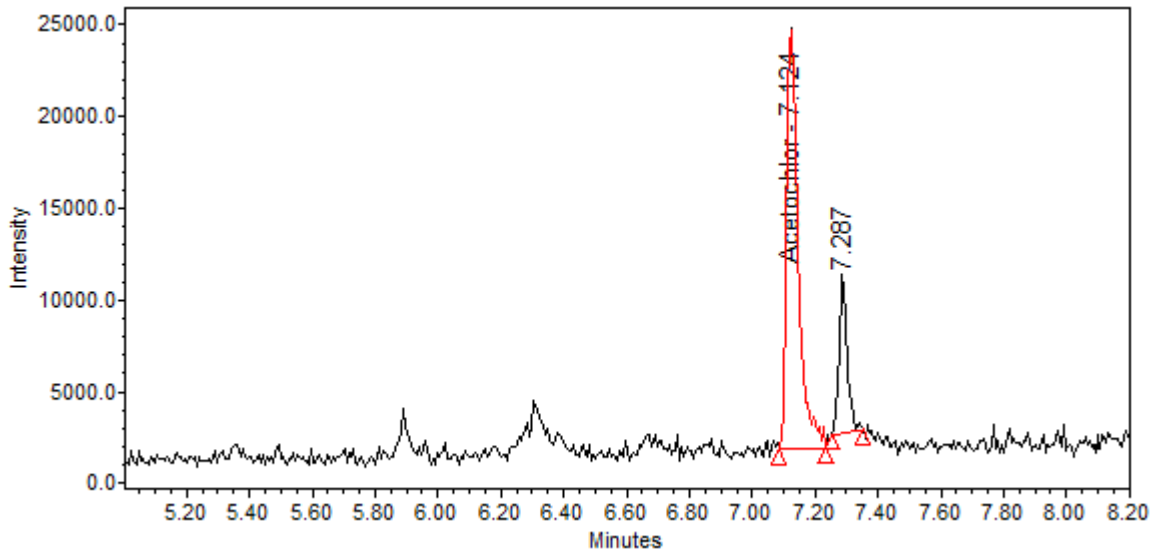


Figure 4.8: SIR chromatogram of acetochlor (m/z 146) at 0.5 µg/mL with a 1 µL injection volume (0.5 ng on column)

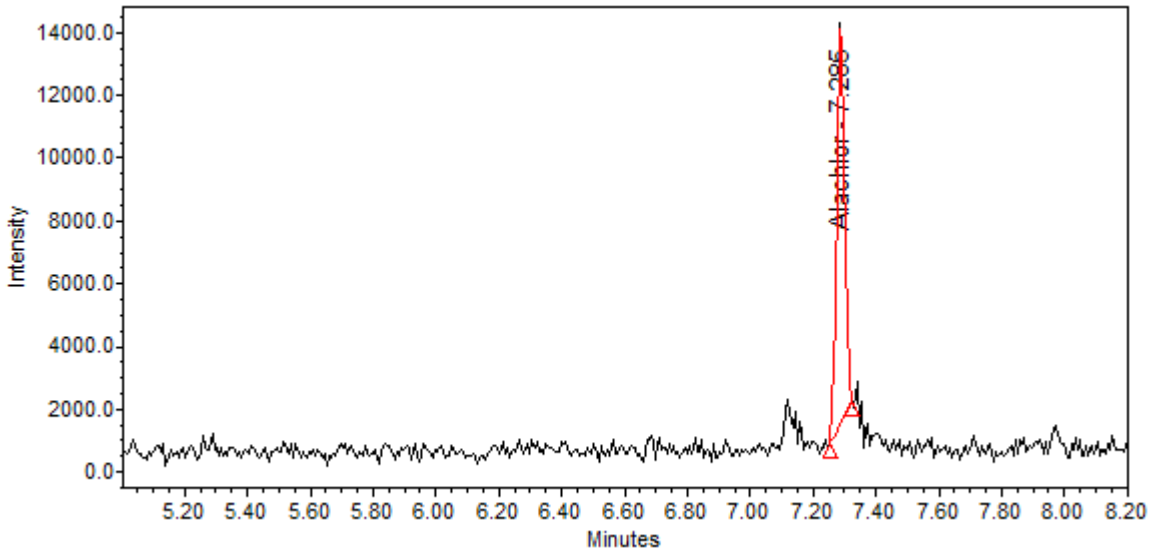


Figure 4.9: SIR chromatogram of alachlor (m/z 188) at 0.5 µg/mL with a 1 µL injection volume (0.5 ng on column)

It is clear that the experimentally determined LOQs are far lower than the calculated concentrations reported in Table 4.11 for all four analytes of interest. Atrazine was found to have the highest LOQ at approximately 0.5 µg/mL.

An injection was also performed at a five times lower concentration (0.1 µg/mL) to further evaluate the LOD and LOQ for each analyte. The signal to noise ratios for each analyte were found to be 5.8 for atrazine (Figure 4.10), 16.6 for terbuthylazine (Figure 4.11), 12.2 for acetochlor (Figure 4.12) and 11.4

for alachlor (Figure 4.13). The S/N values for each analyte were higher than expected, not five times lower like the concentration. The noise was observed to be reduced along with the signal intensity. The concentration of 0.1 µg/mL was, however, found to be approximately at the LOQ for acetochlor and alachlor with their respective S/N values approaching 10. Terbuthylazine was thus found to have the lowest LOQ, under 0.1 µg/mL.

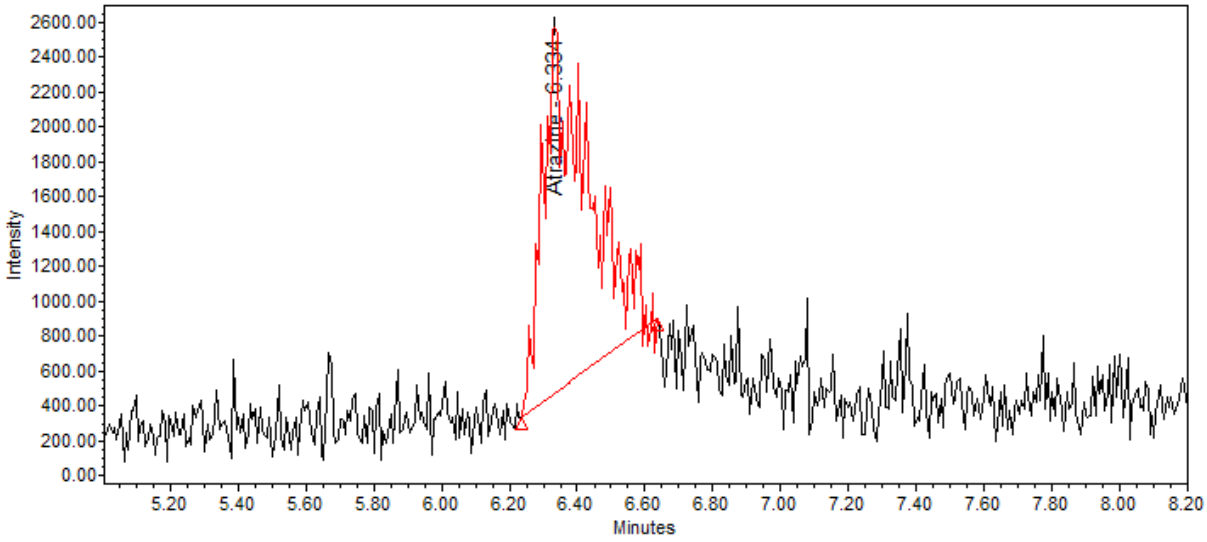


Figure 4.10: SIR chromatogram of atrazine (m/z 200) at 0.1 µg/mL with a 1 µL injection volume (0.1 ng on column)

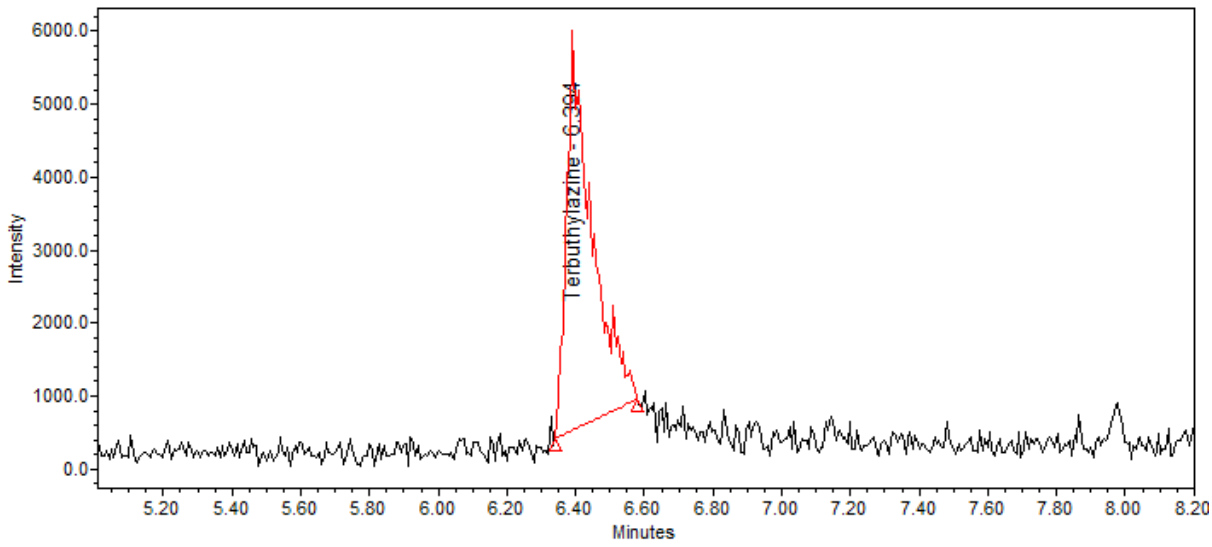


Figure 4.11: SIR chromatogram represting terbuthylazine (m/z 214) at 0.1 µg/mL with a 1 µL injection volume (0.1 ng on column)

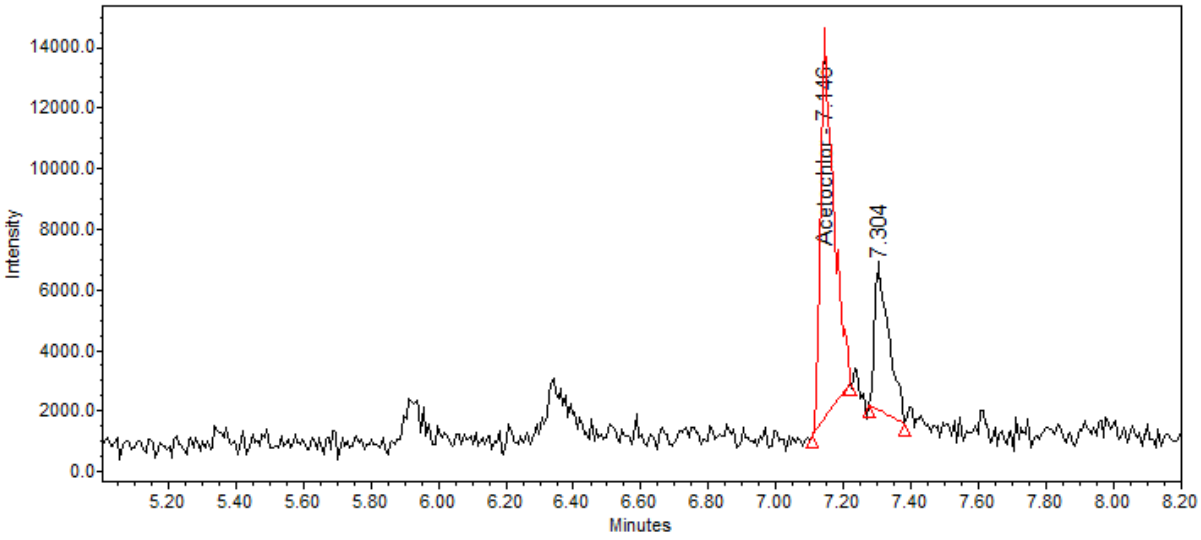


Figure 4.12: SIR chromatogram of acetochlor (m/z 146) at 0.1 µg/mL with a 1 µL injection volume (0.1 ng on column).The second peak is from the mass fragment of alachlor at a m/z of 146

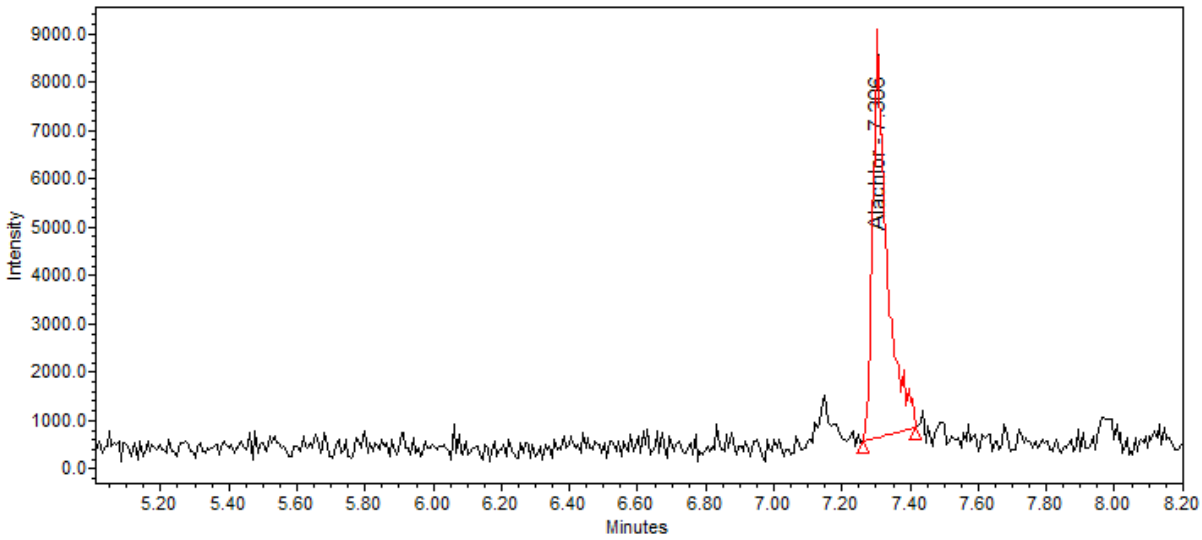


Figure 4.13: SIR chromatogram of alachlor (m/z 188) at 0.1 µg/mL with a 1 µL injection volume (0.1 ng on column)

A final injection was done at 0.05 µg/mL (0.05 ng on column), and this was found to be close to the LOD for terbuthylazine, acetochlor and alachlor. Atrazine was not detected at this concentration as the S/N was found to be under 3. Atrazine (Figure 4.14) was found to have a S/N of 2.5, terbuthylazine (Figure 4.15) had a S/N value of 8.1, acetochlor (Figure 4.16) had a S/N value of 4.9 and alachlor (Figure 4.17) was found to have a S/N value of 4.2.

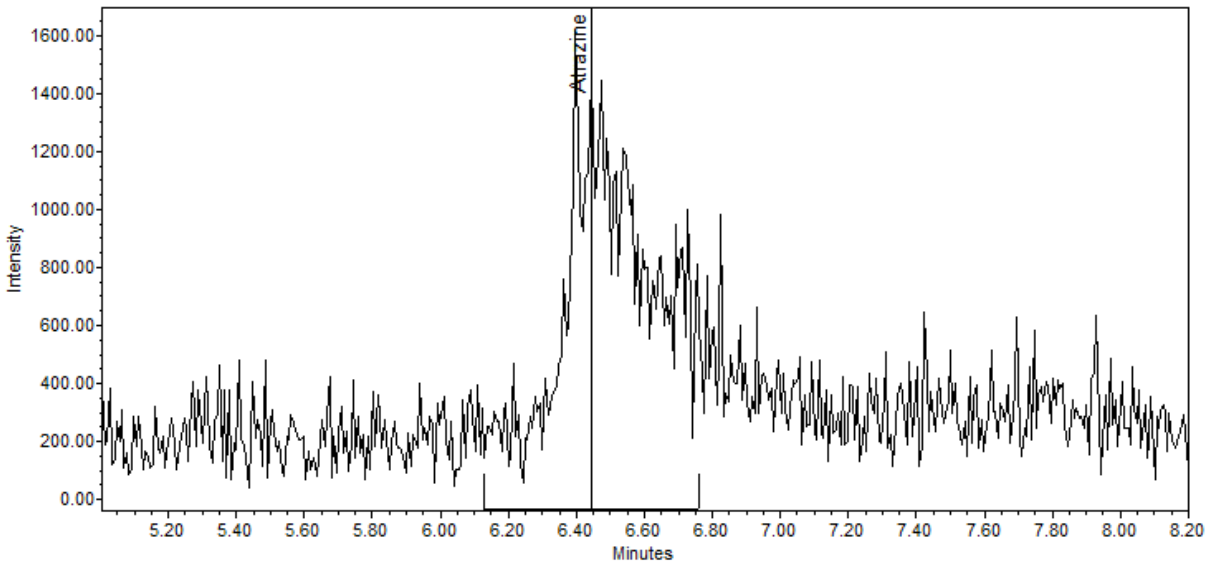


Figure 4.14: SIR chromatogram of the atrazine peak (m/z 200) at 0.05 µg/mL (0.05 ng on column injection)

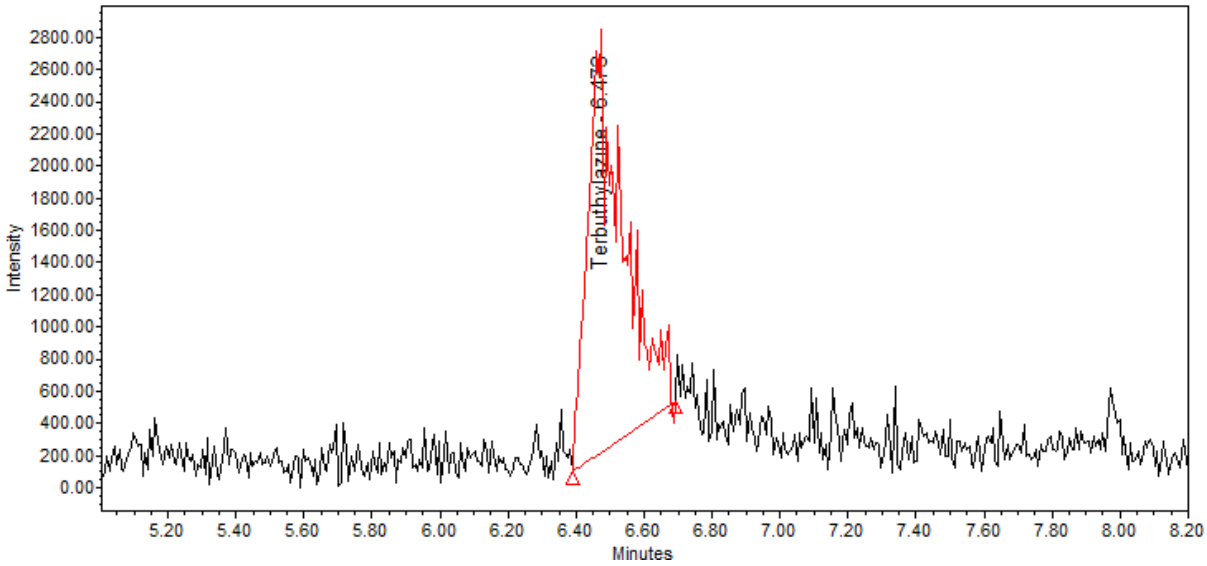


Figure 4.15: SIR chromatogram of the terbuthylazine peak (m/z 214) at 0.05 µg/mL (0.05 ng on column injection)

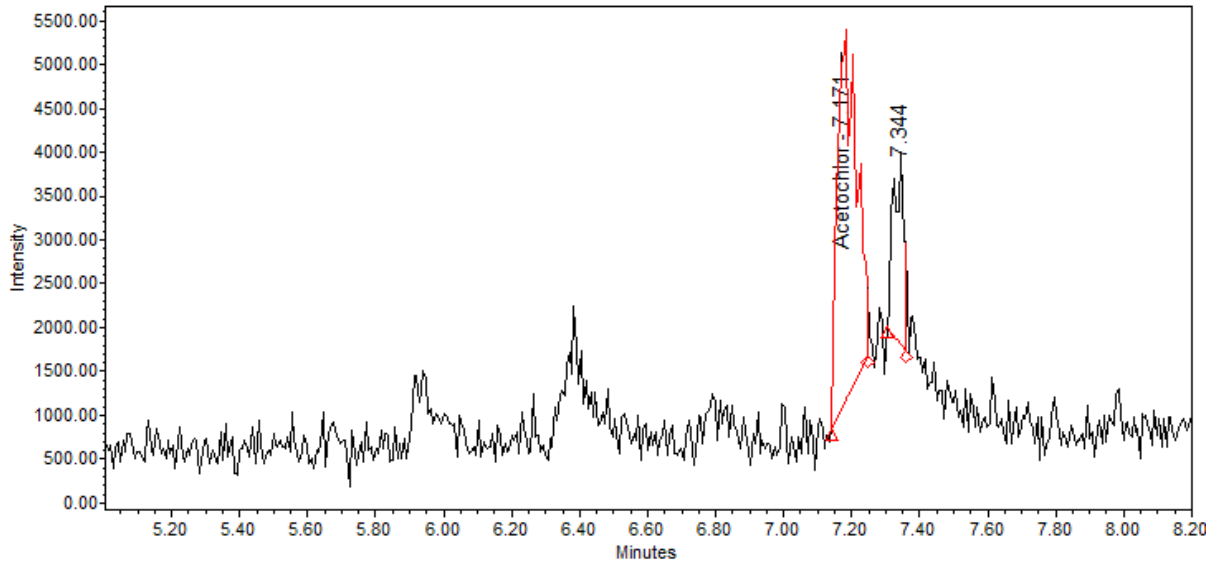


Figure 4.16: SIR chromatogram of the acetochlor peak (m/z 146) at 0.05 µg/mL (0.05 ng on column injection)

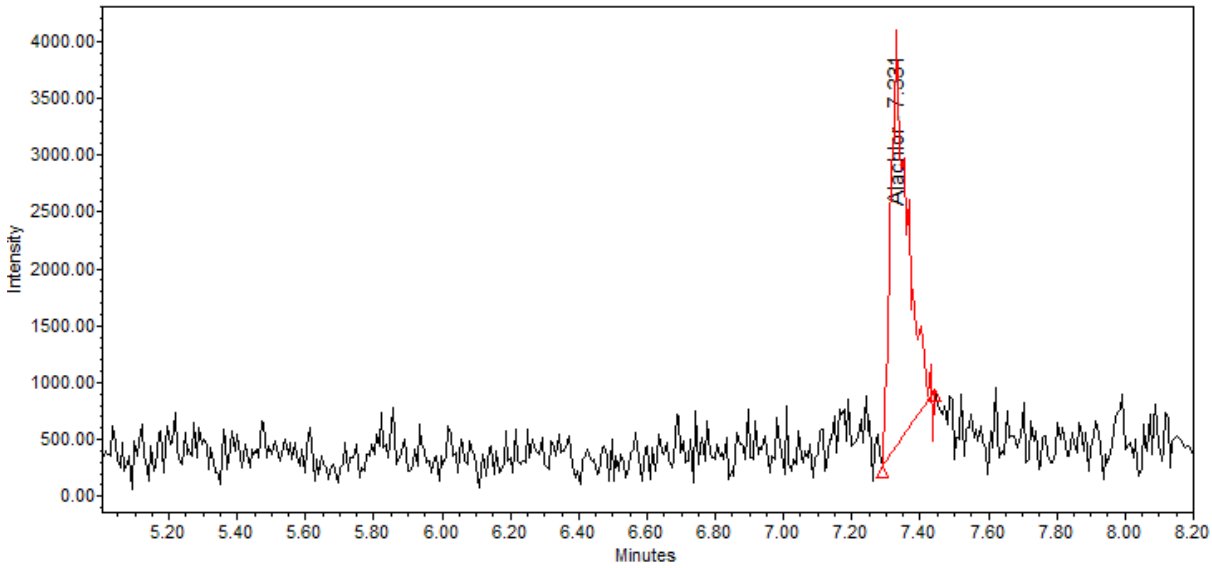


Figure 4.17: SIR chromatogram of the alachlor peak (m/z 188) at 0.05 µg/mL (0.05 ng on column injection)

Inter- and intraday replicates

Data was obtained from three sets of five replicate analyses conducted over two days (Table 4.12). The 3 sets where done in the morning and afternoon of day 1 and the morning of day 2, respectively. Controls were made up on the first calibration point of the calibration curve (2.5 µg/mL) and 5 injections were performed on each day for three days, resulting in a total of 15 injections. The average measured concentration, standard deviation, and subsequent % relative standard deviation (%RSD) were then calculated for each analyte.

Table 4.12: Statistical validation for 15 interday replicate level 1 samples over two days

Set	Rep #	Expected concentration (µg/ mL)	Measured atrazine concentration (µg/ mL)	Measured terbuthylazine concentration (µg/ mL)	Measured acetochlor concentration (µg/ mL)	Measured alachlor concentration (µg/ mL)
Morning day 1	1	2.5	2.52	2.56	2.25	2.98
	2	2.5	2.47	2.61	2.34	2.94
	3	2.5	2.43	2.62	2.21	2.96
	4	2.5	2.41	2.81	2.32	2.89
	5	2.5	2.77	2.85	2.00	2.83
Afternoon day 1	1	2.5	2.57	2.46	2.14	2.72
	2	2.5	2.74	2.32	2.17	2.62
	3	2.5	2.53	2.36	2.19	2.37
	4	2.5	2.47	2.61	2.42	2.76
	5	2.5	2.25	2.44	2.21	2.35
Morning day 2	1	2.5	2.76	2.54	2.51	2.46
	2	2.5	2.29	2.55	2.67	2.42
	3	2.5	2.50	2.78	2.68	2.51
	4	2.5	2.76	2.67	2.21	2.23
	5	2.5	2.34	2.53	2.69	2.68
Average (µg/mL)			2.52	2.58	2.33	2.65
Standard deviation (µg/mL)			0.17	0.15	0.22	0.25
RSD (%)			6.81	5.97	9.22	9.31
Bias (µg/mL)			**0.02**	**0.08**	**-0.17**	**0.15**

The bias was calculated from the data in Table 4.12 by calculating the sum of the difference between each measured value for each analyte and dividing this sum by the total number of measurements (n=15). The %RSDs were all found to be under 10% and each bias was under 0.2 µg/mL. These statistical parameters indicate that the GC-MS method was found to robust enough for between day analysis and was unbiased. Table 4.13 displays the intraday, or rather intra-set comparison, where the average and %RSD were calculated for each set of 5 replicates as displayed in Table 4.12. The expected concentration is also 2.5 µg/mL as in the interday comparison. The %RSDs were all found to be under 10% for the intraday analysis, the bias was higher in some cases than the interday validation data as the averages were found to deviate from 2.5 µg/mL.

Table 4.13: Statistical validation for 15 replicate injections for intraday comparison

Set	Atrazine		Terbuthylazine		Acetochlor		Alachlor	
	Average (µg/mL)	RSD (%)	Average (µg/mL)	RSD (%)	Average (µg/mL)	RSD (%)	Average (µg/mL)	RSD (%)
Morning day 1	2.52	5.79	2.69	4.85	2.22	6.10	2.92	2.07
Afternoon day 1	2.51	7.07	2.44	4.59	2.23	5.01	2.56	7.54
Morning day 2	2.53	8.85	2.61	4.16	2.55	8.03	2.46	6.60

4.2.4: Effect of silanization

An example chromatogram of when the GC inlet liner was found to be active is displayed in Figure 4.18, in which atrazine was observed to tail. In contrast, Figure 4.19 displays the chromatogram obtained just after the inlet liner had been cleaned with methanol and water, and deactivated with 10% DMDCS toluene solution. It is clear that the atrazine peak was much sharper with the silanized inlet liner. Figure 4.20 and Figure 4.21 include some example chromatograms to demonstrate the effect that deactivating the inlet liner had on acetochlor and alachlor, where with the active liner (Figure 4.20) no baseline separation was achieved.

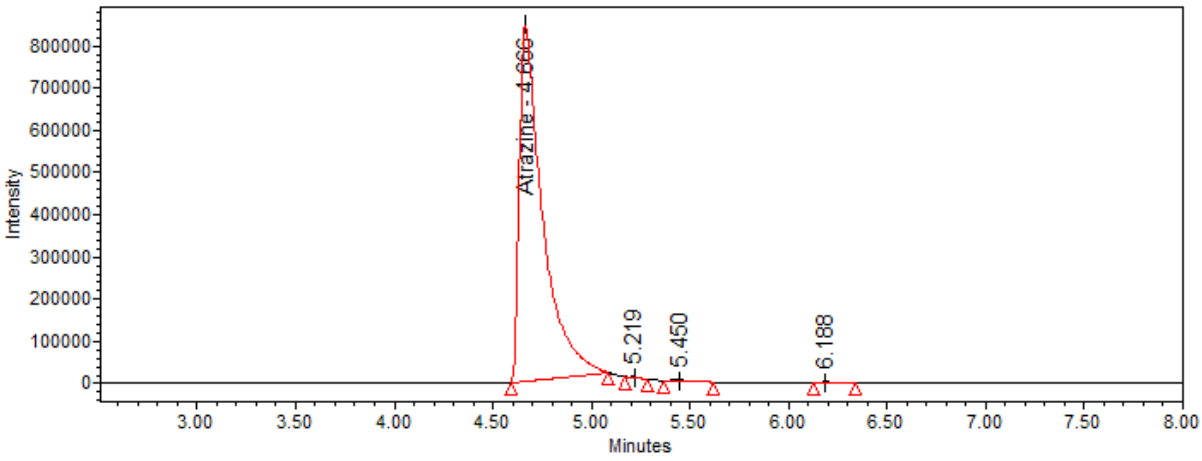


Figure 4.18: An example SIR chromatogram of a tailing atrazine peak, with the inlet liner being active and causing extra retention

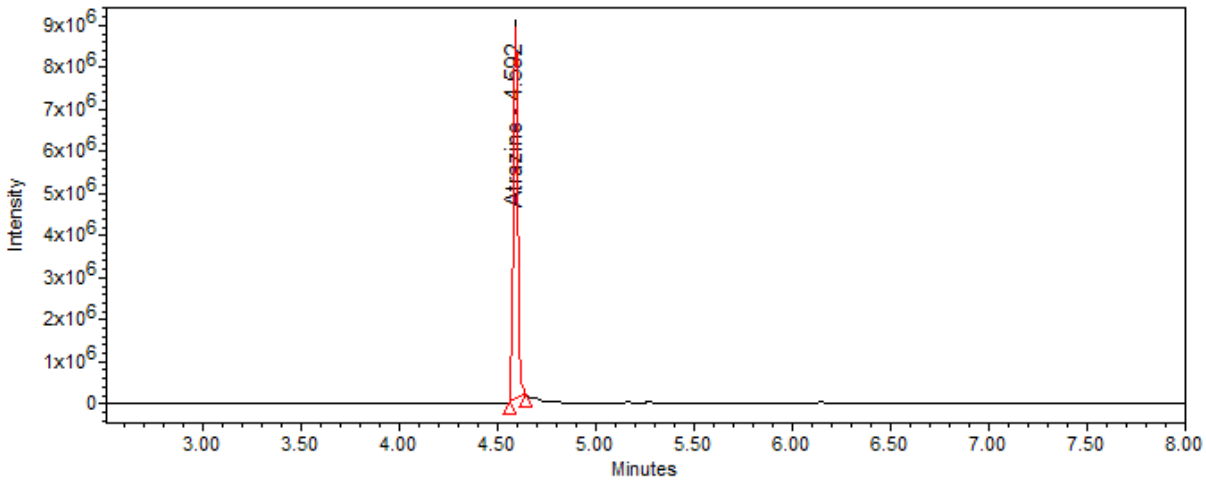


Figure 4.19: An example SIR chromatogram of atrazine with a freshly cleaned and deactivated liner

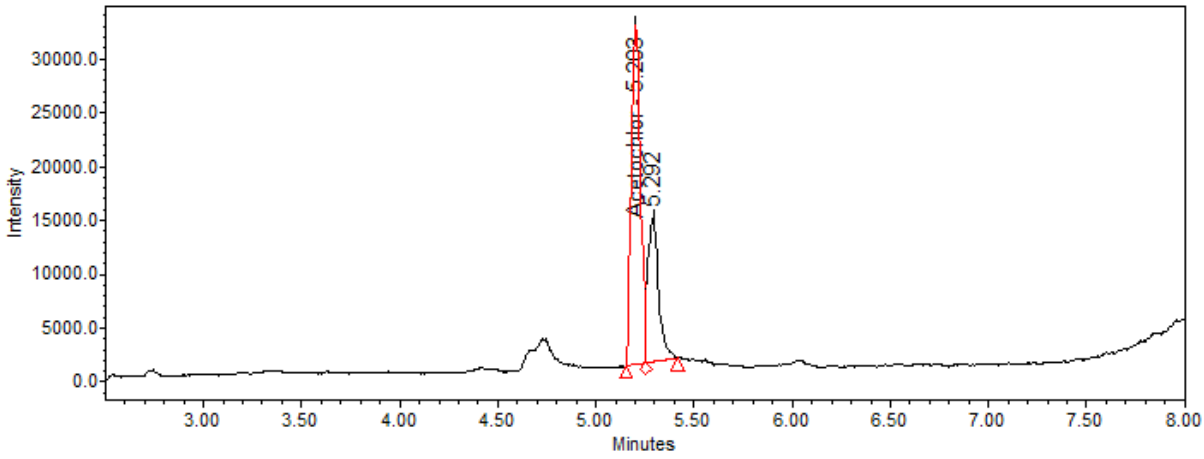


Figure 4.20: An example SIR chromatogram showing no baseline separation between the acetochlor and alachlor peaks with a "dirty" inlet liner

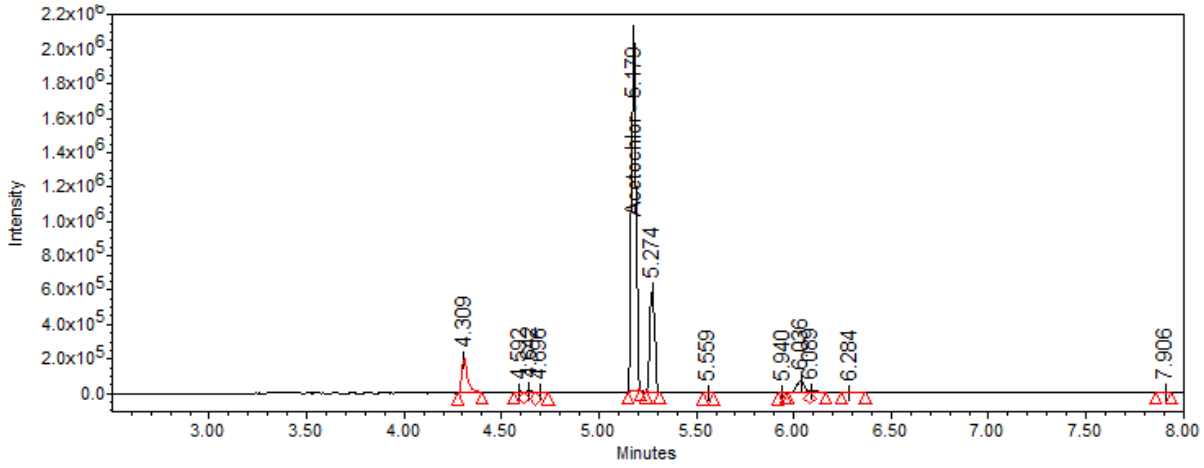


Figure 4.21: Baseline separation of alachlor and acetochlor is obtained in this example SIR chromatogram after the inlet liner had been freshly cleaned and deactivated

4.3: MIP adsorption capacity

In this section, the results obtained for the experiments described in Section 3.3 are discussed with respect to the adsorption capacity for each of the synthesised polymers, including the non-imprinted polymer (NIP).

4.3.1: Individual pesticide adsorption capacities of the NIP

Three repeat analyses were performed in order to determine the NIP adsorption capacity (Table 4.14), in order to perform statistical analysis.

Table 4.14: Adsorption capacity determination of the non-imprinted polymer (NIP)

Replicate	Pesticide	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
1	**Atrazine**	20.0	15.5	4.0	32.5	0.55
	Acetochlor	20.0	15.8	4.0	28.7	0.59
	Terbuthylazine	20.0	14.9	4.0	30.0	0.68
	Alachlor	20.0	14.4	4.0	34.5	0.65
2	**Atrazine**	20.0	15.5	4.0	35.2	0.51
	Acetochlor	20.0	15.9	4.0	32.9	0.50
	Terbuthylazine	20.0	14.1	4.0	38.7	0.61
	Alachlor	20.0	14.4	4.0	29.2	0.77
3	**Atrazine**	20.0	15.5	4.0	35.6	0.51
	Acetochlor	20.0	14.9	4.0	38.7	0.53
	Terbuthylazine	20.0	14.2	4.0	32.4	0.72
	Alachlor	20.0	14.4	4.0	34.5	0.65

Table 4.15 shows the mean adsorption capacity, the calculated standard deviation and subsequent %RSD for each pesticide along with the associated octanol-water partition coefficient.

Table 4.15: Summarized adsorption capacities of each pesticide on the NIP with its corresponding log K_{ow} value (Dalrymple, 2005; López-Roldán *et al.*, 2004; MacBean, 2012)

Pesticide	Log K_{ow}	Mean adsorption capacity (mg/g)	Standard deviation (mg/g)	RSD (%)
Atrazine	2.82	0.52	0.021	5.5
Acetochlor	3.03	0.54	0.036	9.0
Terbuthylazine	3.40	0.67	0.044	8.8
Alachlor	3.52	0.69	0.056	10.8

Figure 2.20 represents a visual representation of the calculated data from Table 4.15 where a correlation can be seen between the octanol-water partition coefficient and the adsorption capacity for each of the pesticides.

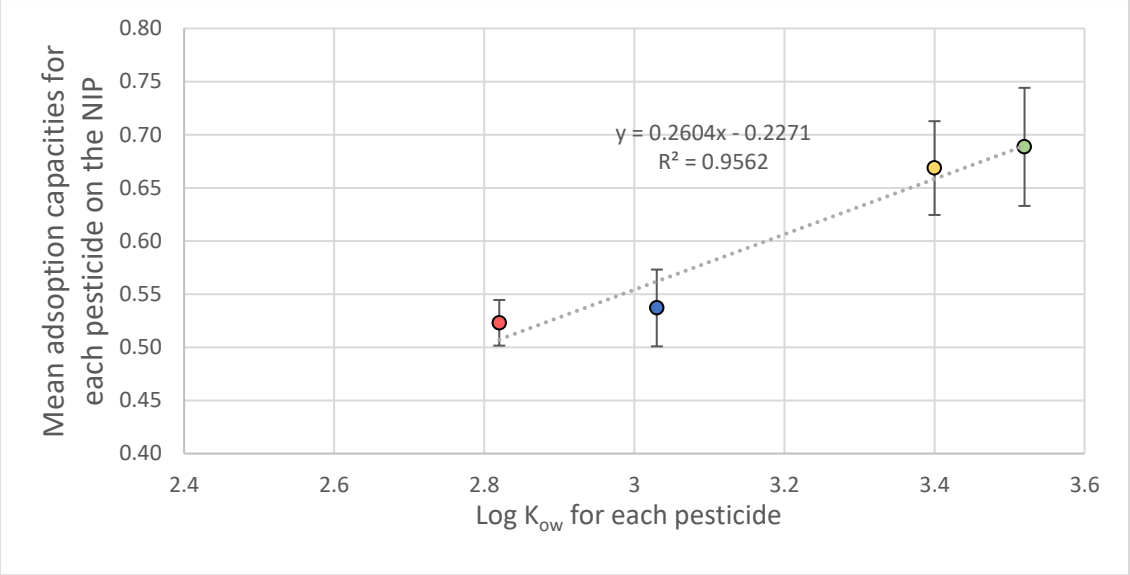


Figure 4.22: The adsorption capacities the of NIP are plotted against the log K_{ow} value for each pesticide. Red represents atrazine, blue represents acetochlor, yellow represents terbuthylazine and green represents alachlor. The error bars are the calculated standard deviations

There is a clear correlation (R^2 = 0.9562) between the adsorption capacity of the selected pesticides on the NIP and their respective log K_{ow} values. This indicates that hydrophobicity plays a large role in the adsorption of pesticides onto the NIP, as the adsorption capacity of NIP increases as the octanol-water partition coefficient increases. Alachlor has the highest log K_{ow} value of 3.52, and consequently has the highest affinity for the NIP with an adsorption capacity of 0.69 mg/g. The same trend was later observed in MTMIP adsorption capacity data, where an almost linear response was observed when comparing the adsorption capacity and log K_{ow} values. The two graphs displayed in Figure 4.22 and Figure 4.27 depict this correlation.

The atrazine NIP adsorption capacity was found to be lower than that reported in literature, where a NIP prepared from the same monomers adsorbed 0.64 µg/g atrazine. The described experiment was however a dynamic experiment, where a 500 mL spiked aqueous sample was passed through a cartridge containing the NIP (Kueseng *et al.*, 2009). The experiment performed in this study was a batch experiment. The NIP adsorption capacity found in this study strongly correlates to that of a reported triazine MIP, where 0.42 mg/g propazine was reported to adsorb to a similar NIP also synthesised from MAA and EDGMA (Geng *et al.*, 2015).

t-test

The paired t-test, as described in Section 3.9.1, was used to statistically compare the NIP adsorption capacities of the different pesticides (Table 4.16). The t critical value is 2.776 at a p-value 0.05 and with 4 degrees of freedom.

Table 4.16: Comparison of the selected pesticide's adsorption capacity on the NIP utilising a t-test

Compared pesticides	Mean A (mg/g)	Mean B (mg/g)	Standard deviation A (mg/g)	Standard deviation B (mg/g)	t (t_{crit}=2.776)
Atrazine (A) vs acetochlor (B)	0,52	0,54	0,02	0,04	0,83
Acetochlor (A) vs terbuthylazine (B)	0,54	0,67	0,04	0,04	3,96
Terbuthylazine (A) vs alachlor(B)	0,67	0,69	0,04	0,06	0,49
Atrazine (A) vs terbuthylazine (B)	0,52	0,67	0,02	0,04	5,33
Acetochlor (A) vs alachlor (B)	0,54	0,69	0,04	0,06	3,90

Table 4.16 indicates no statistical difference between the adsorption capacities of acetochlor and atrazine on the NIP. There is a statistical difference between the adsorption capacities of acetochlor and terbuthylazine, but again, no difference between terbuthylazine and alachlor. There is a statistical difference between terbuthylazine and atrazine. Acetochlor and alachlor were also found to be statistically different in terms of their adsorption capacity to the NIP.

The adsorption capacity on the NIP appears to be more dependent on the log K_{OW} values of each pesticide than the pesticide class as terbuthylazine and alachlor have similar log K_{OW} values but are from different pesticide classes.

4.3.2: Adsorption capacity for individual template MIPs

4.3.2.1: Triazine pesticide class

Table 4.17 displays the calculated adsorption capacity for all four pesticides on the atrazine molecularly imprinted polymer (MIP). All experiments were done in triplicate for statistical analysis.

Table 4.17: Adsorption capacity for each of the four pesticides of interest on the atrazine MIP done in triplicate

Pesticides	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
Atrazine	20.0	13.0	4.0	30.4	**0.92**
Atrazine	20.0	11.5	4.0	34.3	**0.99**
Atrazine	20.0	11.9	4.0	39.4	**0.82**
Terbuthylazine	20.0	9.6	4.0	48.1	**0.86**
Terbuthylazine	20.0	10.0	4.0	42.1	**0.95**
Terbuthylazine	20.0	12.5	4.0	30.2	**0.99**

Pesticides	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
Acetochlor	20.0	16.8	4.0	28.7	**0.45**
Acetochlor	20.0	15.3	4.0	40.3	**0.47**
Acetochlor	20.0	17.2	4.0	35.7	**0.31**
Alachlor	20.0	15.4	4.0	34.5	**0.53**
Alachlor	20.0	16.4	4.0	29.8	**0.48**
Alachlor	20.0	15.3	4.0	33.2	**0.57**

Table 4.18 displays the calculated adsorption capacity for all four pesticides on the terbuthylazine MIP. All experiments were done in triplicate for statistical analysis, however it is clear from the data in Table 4.18 that the triazine pesticides have a higher adsorption capacity on the terbuthylazine MIP.

Table 4.18: Adsorption capacity for each of the four pesticides of interest on the terbuthylazine MIP done in triplicate

Pesticides	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
Atrazine	20	12.8	4	27.4	**1.05**
Atrazine	20	11.3	4	30.9	**1.13**
Atrazine	20	11.7	4	35.5	**0.94**
Terbuthylazine	20	10.8	4	44.8	**0.82**
Terbuthylazine	20	9.8	4	37.9	**1.08**
Terbuthylazine	20	11.4	4	35.3	**0.98**
Acetochlor	20	15.1	4	25.8	**0.68**
Acetochlor	20	13.8	4	36.3	**0.62**
Acetochlor	20	15.5	4	32.1	**0.50**
Alachlor	20	13.9	4	31.1	**0.44**
Alachlor	20	14.8	4	26.8	**0.67**
Alachlor	20	13.8	4	29.9	**0.70**

Table 4.19 contains a summary of the mean adsorption capacity for each of the four pesticides of interest on the atrazine and terbuthylazine MIPs, respectively. The mean adsorption capacity column was colour coded from green (hight values) to red (low values).

Table 4.19: Mean, standard deviation and %RSD for atrazine and terbuthylazine MIP adsorption capacity

Sorbent	Pesticide	Mean adsorption capacity (mg/g)	Standard deviation (mg/g)	RSD (%)
Atrazine MIP	Atrazine	0.91	0.069	7.6
	Terbuthylazine	0.93	0.056	6.0
	Acetochlor	0.60	0.074	12.3
	Alachlor	0.72	0.021	3.0
Terbuthylazine MIP	Atrazine	1.04	0.079	7.6
	Terbuthylazine	0.96	0.105	11.0
	Acetochlor	0.49	0.076	15.4
	Alachlor	0.60	0.117	16.4

Bar graphs were drawn up to allow for easy visual comparison of the adsorption capacities of each respective pesticide on the two triazine MIPs. Error bars are displayed to indicate the calculated standard deviation for the adsorption capacity of each pesticide. Figure 4.23 represents the atrazine MIP and Figure 4.24 the terbuthylazine MIP.

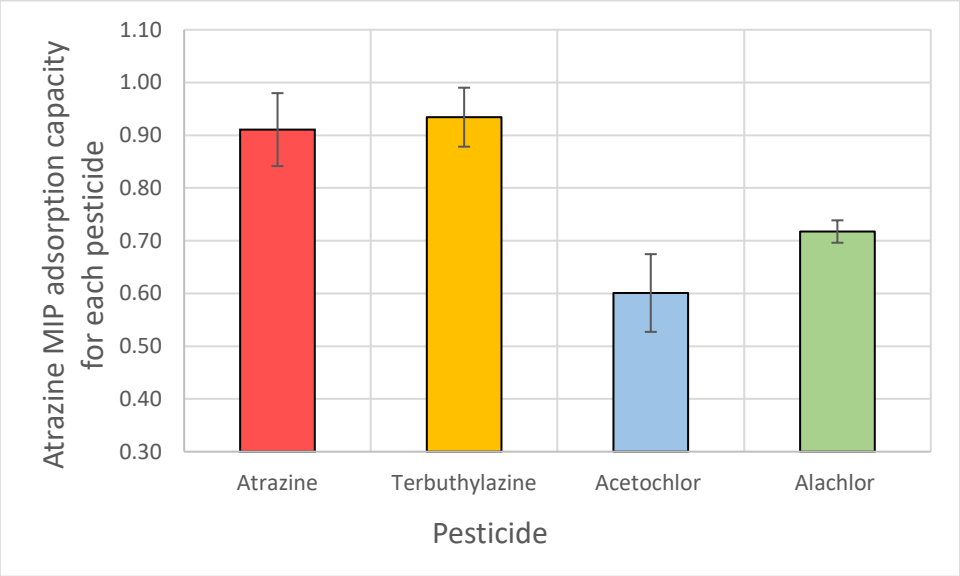


Figure 4.23: Atrazine MIP adsorption capacity for each pesticide with error bars indicating the calculated standard deviation where N=3 for each pesticide analyte

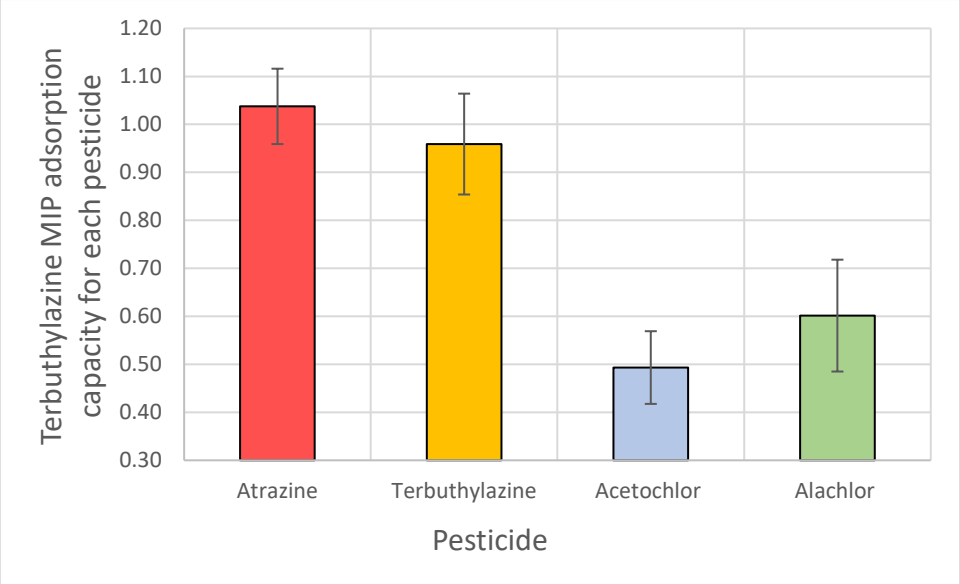


Figure 4.24: Terbuthylazine MIP adsorption capacity for each pesticide with error bars indicating the calculated standard deviation where N=3 for each analyte

t-test

The paired t-test, as described in Section 3.9.1, was used to statistically compare the adsorption capacities of the different pesticides on the triazine MIPs. The t critical value is 2.776 at a p value 0.05 and with 4 degrees of freedom. Table 4.20 contains the results of the t-test to compare the different adsorption capacities of the triazine pesticides on the triazine MIPs.

Table 4.20: T-test of adsorption capacities of the triazine pesticides on the triazine MIPs

Compared groups	Mean A (mg/g)	Mean B (mg/g)	Standard deviation A (mg/g)	Standard deviation B (mg/g)	t (t_{crit}=2.776)
Atrazine (A) vs terbuthylazine (B) on atrazine MIP	0.91	0.93	0.069	0.056	0.46
Atrazine (A) vs terbuthylazine (B) on terbuthylazine MIP	1.04	0.96	0.079	0.105	1.04

There was no statistical difference between the binding capacity of atrazine or terbuthylazine on either the atrazine or terbuthylazine MIP. This is because of the shapes of the cavities and the orientation of functional hydrogen bonding groups in the MIPs being similar to one another. Refer to Section 2.7.2.3 for schematic representations of the self-orientation of the functional monomers. Section 2.7.1. discusses the use of dummy templates in literature where a similar observation was made in terms of shared affinity for a structurally similar compound. Table 4.21 displays a series of t-tests for comparison of the adsorption capacities of the pesticides to the triazine MIPs and NIP, respectively.

Table 4.21: Adsorption capacity t-test to compare the NIP to triazine MIPs

Compared groups	Mean A (mg/g)	Mean B (mg/g)	Standard deviation A (mg/g)	Standard deviation B (mg/g)	t (t_{crit}=2.776)
Atrazine on atrazine MIP (A) vs atrazine on NIP (B)	0.91	0.52	0.069	0.021	**9.27**
Terbuthylazine on atrazine MIP (A) vs terbuthylazine on NIP (B)	0.93	0.67	0.056	0.036	**6.46**
Acetochlor on atrazine MIP (A) vs acetochlor on NIP (B)	0.60	0.69	0.068	0.044	1.35
Alachlor on atrazine MIP (A) vs alachlor on NIP (B)	0.72	0.67	0.034	0.056	0.84
Atrazine on terbuthylazine MIP (A) vs atrazine on NIP (B)	1.04	0.52	0.079	0.021	**13.72**
Terbuthylazine on terbuthylazine MIP (A) vs terbuthylazine on NIP (B)	0.96	0.67	0.105	0.036	**6.95**
Acetochlor on terbuthylazine MIP (A) vs acetochlor on NIP (B)	0.49	0.69	0.076	0.044	0.90
Alachlor on terbuthylazine MIP (A) vs alachlor on NIP (B)	0.60	0.67	0.117	0.056	1.17

There is a clear difference in adsorption capacity of the two triazine pesticides on the NIP and their adsorption capacities on triazine MIPs. There is no statistical difference between the adsorption capacity of the two chloroacetanilide pesticides on triazine MIPs and the NIP. This proves the selectivity of triazine MIPs for triazine pesticides. The alachlor and acetochlor adsorb no more to the triazine MIPs than to the NIP, thus indicating they only adsorb by hydrophobic interactions and coincidental hydrogen bonding because they do not compliment the shape of the cavities and the subsequent functional monomer orientation of the triazine MIPs.

The obtained adsorption capacities strongly correlate to reported literature which reports 1.00 mg/g adsorption capacity of atrazine on a similar MIP (Kueseng *et al.*, 2009). Another literature example reported quite different values for a similar propazine MIP on a TiO_2 surface, where 6.81 mg/g propazine was adsorbed to the MIP, indicating that the TiO_2 might have had a significant effect on the triazine pesticide adsorption capacity to the MIP (Geng *et al.*, 2015).

4.3.2.2: Chloroacetanilide pesticide class

Table 4.22 and 4.23 display the calculated adsorption capacities for all four pesticides on the acetochlor and alachlor MIPs respectively. All experiments were done in triplicate for statistical analysis. The methodology was described in Section 3.3.2.

Table 4.22: Binding capacity for each of the four pesticides of interest on the acetochlor MIP done in triplicate

Pesticides	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
Atrazine	20.0	15.3	4.0	37.6	0.50
Atrazine	20.0	13.9	4.0	39.9	0.61
Atrazine	20.0	16.9	4.0	25.1	0.49
Terbuthylazine	20.0	14.8	4.0	29.0	0.72
Terbuthylazine	20.0	15.5	4.0	27.5	0.65
Terbuthylazine	20.0	16.1	4.0	30.5	0.51
Acetochlor	20.0	7.9	4.0	45.0	1.08
Acetochlor	20.0	10.9	4.0	40.9	0.89
Acetochlor	20.0	11.3	4.0	30.1	1.16
Alachlor	20.0	13.3	4.0	30.1	0.89
Alachlor	20.0	11.5	4.0	34.0	1.00
Alachlor	20.0	10.2	4.0	35.7	1.10

Table 4.23: Binding capacity for each of the four pesticides of interest on the alachlor MIP done in triplicate

Pesticides	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
Atrazine	20.0	15.4	4.0	36.6	0.50
Atrazine	20.0	13.9	4.0	39.9	0.61
Atrazine	20.0	16.9	4.0	25.1	0.49
Terbuthylazine	20.0	14.8	4.0	29.0	0.72
Terbuthylazine	20.0	15.5	4.0	27.5	0.65
Terbuthylazine	20.0	16.1	4.0	30.5	0.51
Acetochlor	20.0	12.4	4.0	31.6	0.96
Acetochlor	20.0	10.9	4.0	35.7	1.02
Acetochlor	20.0	11.3	4.0	40.9	0.85
Alachlor	20.0	13.3	4.0	30.1	0.89
Alachlor	20.0	11.5	4.0	30.1	1.13
Alachlor	20.0	10.2	4.0	34.0	1.15

From Table 4.22 and 4.23, the mean adsorption capacity, standard deviation and subsequent %RSD were calculated and are represented in Table 4.24. The mean adsorption capacity column in Table 4.24 was colour coded from green (high value) to red (low value).

Table 4.24: Mean, standard deviation and %RSD for pesticide adsorption capacity on the chloroacetanilide MIPs

Sorbent	Pesticide	Mean adsorption capacity (mg/g)	Standard deviation (mg/g)	RSD (%)
Acetochlor MIP	Atrazine	0.54	0.054	10.1
	Terbuthylazine	0.63	0.086	13.7
	Acetochlor	1.04	0.112	10.7
	Alachlor	1.00	0.085	8.5
Alachlor MIP	Atrazine	0.54	0.053	10.0
	Terbuthylazine	0.63	0.086	13.7
	Acetochlor	0.94	0.070	7.4
	Alachlor	1.06	0.119	11.2

The following two bar graphs (Figure 4.24 and 4.25) were plotted using the calculated mean for the adsorption capacity of each of the four pesticide of interest on the acetochlor and alachlor MIPs respectively. The standard deviation is depicted as error bars on each of the bar graphs.

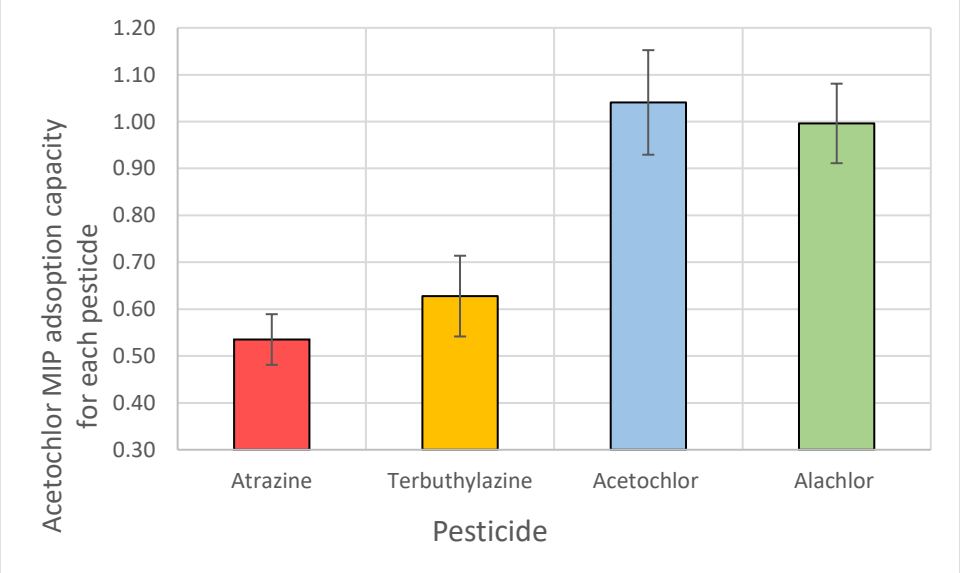


Figure 4.25: Acetochlor MIP adsorption capacity for each pesticide with error bars indicating the calculated standard deviation with N=3 for each analyte

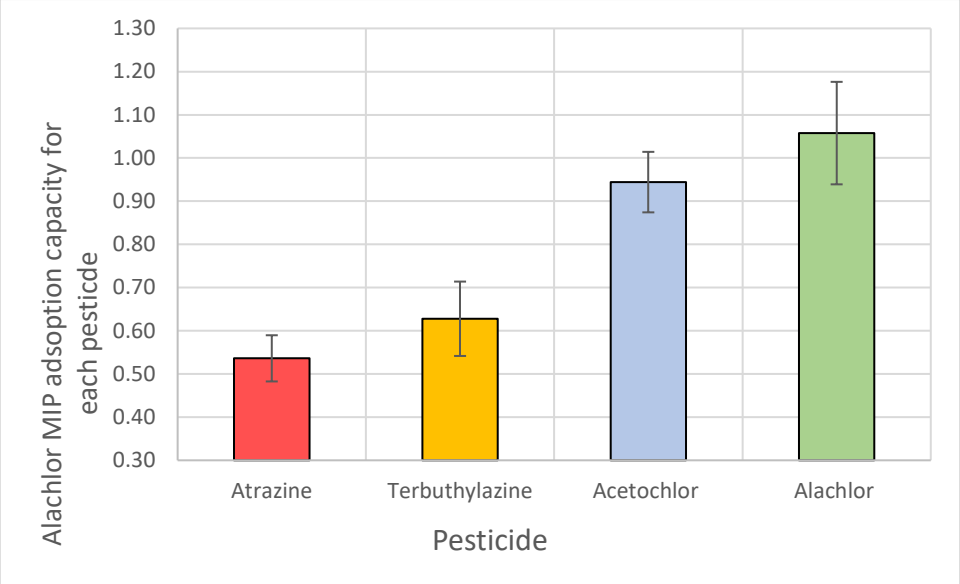


Figure 4.26: Alachlor MIP adsorption capacity for each pesticide with error bars indicating the calculated standard deviation with N= 3 for each analyte

t-test

The paired t-test, as described in Section 3.9.1, was used to statistically compare the adsorption capacities for the different pesticides on the chloroacetanilide MIPs, refer to Table 4.25. The t critical value is 2.776 at a p-value 0.05 and with 4 degrees of freedom.

Table 4.25: Adsorption capacities of chloroacetanilide MIP compared using the t-test

Compared groups	Mean A (mg/g)	Mean B (mg/g)	Standard deviation A (mg/g)	Standard deviation A (mg/g)	t (t_{crit}=2.776)
Acetochlor (A) vs alachlor (B) on acetochlor MIP	1.04	1.00	0.112	0.085	0.55
Acetochlor (A) vs alachlor (B) on alachlor MIP	0.94	1.06	0.070	0.119	1.43

There was no statistical difference found between the binding capacity of acetochlor or alachlor pesticides on either the acetochlor or alachlor MIPs. This is due to the similar cavity shape and monomer functional group orientation in these cavities such that either of the chloroacetanilide MIPs has affinity for either of the chloroacetanilide pesticides. Refer to Section 2.7.2.3 for schematic representations of the monomer self-orientation. A further t-test was performed comparing the adsorption capacity of the pesticides to the NIP and chloroacetanilide MIPs respectively (Table 4.26).

Table 4.26: T-test to compare the pesticide adsorption capacity for the NIP to chloroacetanilide MIPs for all four pesticides of interest

Compared groups	Mean A (mg/g)	Mean B (mg/g)	Standard deviation A (mg/g)	Standard deviation B (mg/g)	t (t_{crit}=2.776)
Atrazine on acetochlor MIP (A) vs atrazine on NIP (B)	0.54	0.52	0.054	0.021	0.36
Terbuthylazine on acetochlor MIP (A) vs terbuthylazine on NIP (B)	0.63	0.67	0.086	0.044	0.73
Acetochlor on acetochlor MIP (A) vs acetochlor on NIP (B)	1.04	0.54	0.112	0.036	**7.44**
Alachlor on acetochlor MIP (A) vs alachlor on NIP (B)	1.00	0.69	0.085	0.056	**5.25**
Atrazine on alachlor MIP (A) vs atrazine on NIP (B)	0.54	0.52	0.053	0.021	0.39
Terbuthylazine on alachlor MIP (A) vs terbuthylazine on NIP (B)	0.63	0.67	0.086	0.044	0.73
Acetochlor on alachlor MIP (A) vs acetochlor on NIP (B)	0.94	0.54	0.070	0.036	**8.95**
Alachlor on alachlor MIP (A) vs alachlor on NIP (B)	1.06	0.69	0.119	0.056	**4.88**

From Table 4.26 there was a clear difference in adsorption capacity of the two chloroacetanilide pesticides on either of the chloroacetanilide MIPs when comparing their binding capacities to those on the NIP. There was no statistical difference found between the binding capacity of the two triazine pesticides on chloroacetanilide MIPs and the NIP. This proves the selectivity of the chloroacetanilide MIPs for chloroacetanilide pesticides. The atrazine and terbuthylazine pesticides adsorb no more to the chloroacetanilide MIPs than they did to the NIP, thus indicating they only adsorb by hydrophobic interactions and random hydrogen bonding with the functional monomers, in contrast to the orientated specific hydrogen bonding of the chloroacetanilide pesticides to their complementary cavities in the MIPs.

4.3.3: Adsorption capacity of the enhanced adsorption capacity MIPs

The enhanced adsorption capacity (EAC) MIPs were produced using four times more template molecule than the previously discussed MIPs in Section 4.3.2, thus theoretically four times more cavities should be available for template pesticides, leading to an increase in adsorption capacity by a factor of 4. The obtained adsorption capacities for the pesticides on the EAC MIPs are summarized in Table 4.27. Each experiment was repeated three times for statistical analysis. Refer to Section 3.3 for the experimental procedures followed to obtain the reported results.

Table 4.27: Adsorption capacity determination of the enhanced adsorption capacity MIPs

Sorbent	Pesticide	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
Atrazine EAC MIP	Atrazine	20.0	10.5	4.0	28.7	1.32
	Atrazine	20.0	8.5	4.0	35.2	1.31
	Atrazine	20.0	8.6	4.0	38.9	1.17
Terbuthylazine EAC MIP	Terbuthylazine	20.0	10.3	4.0	25.6	1.52
	Terbuthylazine	20.0	12.2	4.0	24.3	1.28
	Terbuthylazine	20.0	9.8	4.0	27.2	1.50
Acetochlor EAC MIP	Acetochlor	20.0	13.0	4.0	19.1	1.48
	Acetochlor	20.0	9.7	4.0	29.8	1.38
	Acetochlor	20.0	8.5	4.0	35.7	1.29
Alachlor EAC MIP	Alachlor	20.0	10.4	4.0	30.1	1.28
	Alachlor	20.0	11.2	4.0	25.7	1.37
	Alachlor	20.0	10.7	4.0	32.8	1.13

From Table 4.27 the mean adsorption capacity, standard deviation and subsequent %RSD were calculated for each set of adsorption capacity replicates and are displayed in Table 4.28. The adsorption capacity was colour coded, where green indicates a higher value than red.

Table 4.28: Statistical analysis of the adsorption capacity for each selected pesticide on its respective EAC MIP

Sorbent	Pesticide	Mean adsorption capacity (mg/g)	Standard deviation (mg/g)	RSD (%)
Atrazine EAC MIP	Atrazine	1.27	0.066	5.2
Terbuthylazine EAC MIP	Terbuthylazine	1.43	0.106	7.4
Acetochlor EAC MIP	Acetochlor	1.38	0.077	5.6
Alachlor EAC MIP	Alachlor	1.26	0.097	7.7

From Table 4.28, all the mean adsorption capacities were higher than the previously discussed MIPs in Section 4.3.2. Table 4.29 summarises a t-test that was performed to compare the adsorption capacities of the EAC MIPs with standard MIPs (Section 4.3.2).

Table 4.29: Statistical t-test comparison of the EAC MIP with the standard MIPs

Pesticide	Mean A (mg/g) EAC MIP	Mean B (mg/g) standard MIP	Standard deviation A (mg/g) EAC MIP	Standard deviation B (mg/g) standard MIP	t (t_{crit}=2.776)
Atrazine	1.27	0.91	0.066	0.069	**4.17**
Terbuthylazine	1.43	0.96	0.106	0.105	**3.81**
Acetochlor	1.38	1.04	0.077	0.112	**2.97**
Alachlor	1.26	1.06	0.097	0.119	1.48

The t-test revealed a statistical difference ($t > t_{crit}$) between the adsorption capacities of the EAC MIP and standard MIPs for all the template pesticides and their complimentary MIPs with alachlor as the exception. The calculated t value for acetochlor was found to be close to the t_{crit} value, indicating a marginal statistical difference. Both triazine MIPs had a much more distinct statistical difference between the standard MIP and EAC MIP adsorption capacities, as indicated by the larger calculated t value. This may indicate the triazine MIPs might be more receptive to the cavities in the MIPs than the chloroacetanilide pesticides, as they have more sites for orientated hydrogen bonding with the monomers (Section 2.7.2.3). Although the average adsorption capacity for alachlor was larger on the EAC MIP than the standard MIP, the t-test revealed no statistical difference. Acetochlor and alachlor may have been less influenced by the increase in template because there is less opportunity for hydrogen bonding and subsequent monomer self-orientation to form active cavities during the MIP synthesis. Refer to Section 2.7.2.3 for the schematic representations of the self-orientating monomers around acetochlor and alachlor templates.

As expected, increasing the template during MIP synthesis did increase the adsorption capacity because there are more cavities left in the synthesised MIP. For all four templates studied, increasing the template quantity during synthesis increased the adsorption capacity of the MIP as the average adsorption capacity was higher for the EAC MIP than the standard MIP, although the increase in adsorption capacity was nowhere near the expected stoichiometric four times increase. This may be due to a spatial limitation, in other words no more cavities could fit on the available the surface area of the MIP, or that much of the additional template was completely encapsulated inside the MIP structure not allowing the template to be removed or providing inaccessible binding sites. It may also be due to a time limitation in that the monomers did not have enough time to self-orientate in the solution before they were entrained in the polymerization reaction, and much of the template molecules never formed cavities in the MIP.

4.3.4: Multi template MIP adsorption capacity

The results for the multi template (MT) MIP adsorption capacity experiments, from Section 3.3.4, are presented and discussed in this section. Three replicates were performed for each separate pesticide. Table 4.30 shows the data for all the replicates.

Table 4.30: Adsorption capacity determination for each pesticide on the multi template MIP

Replicate	Pesticide	C_0 (mg/L)	C_e (mg/L)	V (mL)	W (mg)	Adsorption capacity (mg/g)
1	Alachlor	20.0	13.9	4.0	33.5	0.73
	Acetochlor	20.0	13.7	4.0	34.3	0.74
	Terbuthylazine	20.0	12.0	4.0	39.4	0.81
	Atrazine	20.0	9.6	4.0	48.1	0.86
2	Alachlor	20.0	13.5	4.0	42.1	0.62
	Acetochlor	20.0	14.7	4.0	30.2	0.70
	Terbuthylazine	20.0	12.8	4.0	35.4	0.82
	Atrazine	20.0	11.0	4.0	40.3	0.89
3	Alachlor	20.0	13.5	4.0	35.7	0.72
	Acetochlor	20.0	14.1	4.0	32.9	0.72
	Terbuthylazine	20.0	14.5	4.0	29.8	0.74
	Atrazine	20.0	13.5	4.0	33.2	0.78

From the replicates in Table 4.30 the mean adsorption capacity, standard deviation and %RSD were subsequently calculated for each template pesticide on the MTMIP (Table 4.31). The mean adsorption capacity was colour coded from green (high value) to red (low value).

Table 4.31: Mean, standard deviation and %RSD for the adsorption capacity of each pesticide on the MTMIP

Pesticide	Mean adsorption capacity (mg/g)	Standard deviation (mg/g)	RSD (%)
Atrazine	0.84	0.052	7.53
Acetochlor	0.72	0.018	2.49
Terbuthylazine	0.79	0.036	4.58
Alachlor	0.69	0.048	5.65

From the data obtained in Table 4.31 a bar graph (Figure 4.27) was drawn up for a visual comparison of the adsorption capacity for each pesticide on the MTMIP. It is interesting to note the differences in adsorption capacity that were unexpected since stoichiometrically an equal amount of template was added for each pesticide. Since atrazine has the highest adsorption capacity on the MTMIP, clearly the correlation between the log K_{OW} and adsorption capacity found for the NIP was not observed here. This indicates clearly that the orientated hydrogen bonds in the cavities of the MIP now play a more important role than hydrophobic interactions.

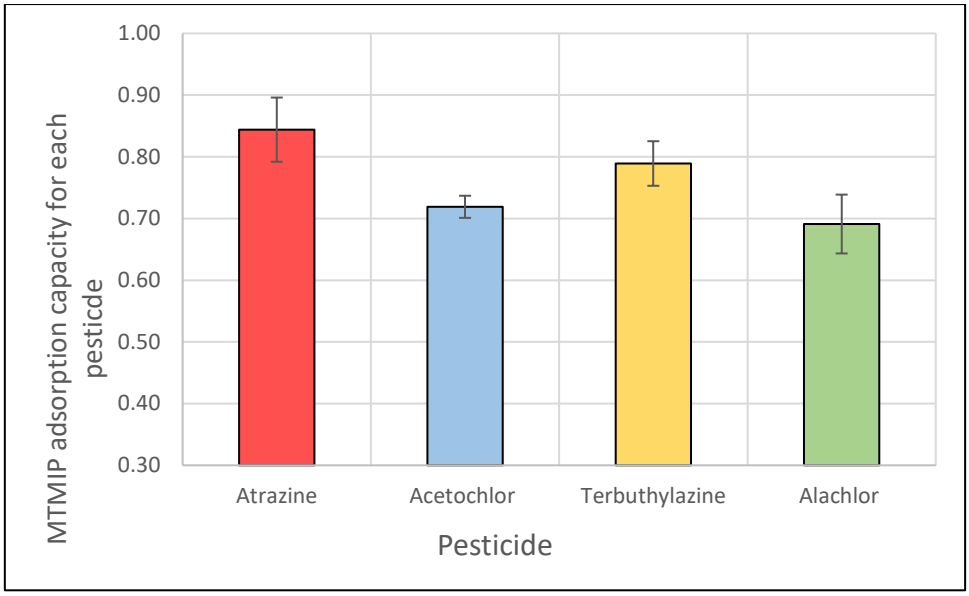


Figure 4.27: Adsorption capacity for each pesticide on the MTMIP where N=3 for each analyte

The bar graph (Figure 4.27) clearly indicates that atrazine and terbuthylazine were found to have higher adsorption capacities on the MTMIP than the chloroacetanilide pesticides. This may be due to more hydrogen bonding and subsequent easier cavity formations for the triazine templates (refer to Section 2.7.2.3). From the standard deviations indicated in Figure 4.27 there may be some overlap, and since the same molar ratio of pesticides were added to the MTMIP during synthesis (Section 3.1.5) there is no expected difference in the number of cavities. The physicochemical properties (Section 2.2.4) of each pesticide reveals an alternative explanation, where acetochlor and alachlor have a much higher water solubility than atrazine and terbuthylazine, which may have influenced the template interactions with the monomer during synthesis.

t-test

The paired t-test, as described in Section 3.9.1, was used to statistically compare the adsorption capacities for the different pesticides on the MTMIP (Table 4.32). The t critical is 2.776 at a p value 0.05 and with degrees of freedom (n) of 4.

Table 4.32: Paired t-test for the comparison of adsorption capacities for the pesticides on the MTMIP

Compared pesticides	Mean A (mg/g)	Mean B (mg/g)	Standard deviation A (mg/g)	Standard deviation B (mg/g)	t (t_{crit}=2.776)
Atrazine (A) vs Terbuthylazine (B)	0.84	0.79	0.052	0.036	1.49
Acetochlor (A) vs terbuthylazine	0.72	0.79	0.018	0.036	**3.02**
Terbuthylazine vs alachlor (B)	0.79	0.69	0.036	0.048	**2.84**
Acetochlor (A) vs alachlor (B)	0.72	0.69	0.018	0.048	0.93

Compared pesticides	Mean A (mg/g)	Mean B (mg/g)	Standard deviation A (mg/g)	Standard deviation B (mg/g)	t (t_{crit}=2.776)
Atrazine (A) vs alachlor (B)	0.84	0.69	0.052	0.048	**3.78**
Atrazine (A) vs acetochlor (B)	0.84	0.72	0.052	0.036	**3.99**

Table 4.32 indicates no statistically significant difference between the adsorption capacities of terbuthylazine and atrazine on the MTMIP. There is a statistical difference between the adsorption capacities of acetochlor and terbuthylazine, and again, a difference between terbuthylazine and alachlor. The chloroacetamide pesticides again have no difference in their adsorption capacities to the MTMIP.

The t-test was also performed for each pesticide class, to compare the NIP and MTMIP (Table 4.33), the t-critical is 2.776 at a p-value 0.05 and with 4 degrees of freedom.

Table 4.33: Paired t-test for NIP and MTMIP adsorption capacities

Compared template pesticides	Mean NIP (A) (mg/g)	Mean MTMIP (B) (mg/g)	Standard deviation (A) NIP (mg/g)	Standard deviation (B) MTMIP (mg/g)	t (t_{crit}=2.776)
Atrazine	0.52	0.69	0.021	0.052	**9.2**
Acetochlor	0.54	0.72	0.036	0.018	**13.6**
Terbuthylazine	0.67	0.79	0.044	0.036	**8.7**
Alachlor	0.69	0.84	0.056	0.048	**7.8**

From the t-test, the adsorption capacities are thus significantly different for all the pesticides when the NIP and MTMIP are compared ($t > t_{crit}$). This proves a higher affinity for the templates to the MTMIP than the NIP and demonstrates again that the orientated hydrogen bonds in the cavities do play a role in increasing the adsorption capacity of the MIPs.

4.4: Polymer characterization

In this section characterization results for the synthesised polymers are presented and discussed.

4.4.1: SEM images

A SEM micrograph of a non-imprinted polymer (NIP), before size fractionation (Figure 4.28), reveals that the polymer is made up of beads of about 4 µm in diameter. There appears to be large conglomerates of these small beads with diameters of 65 µm and larger.

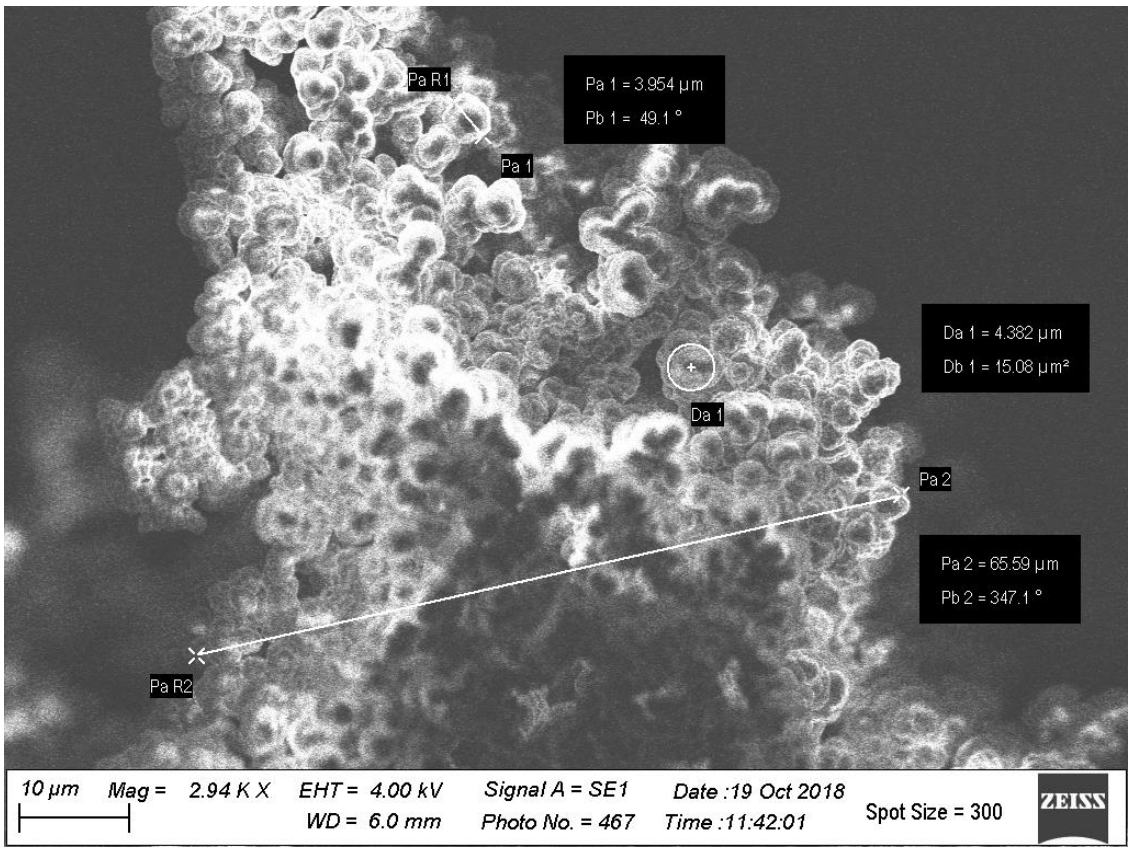


Figure 4.28: SEM micrograph of a non-imprinted polymer (NIP) mounted on a conductive carbon tab before any size fractionation was performed

Using ImageJ software, the average diameter of the particles was determined to be 3.87 µm for N = 30 and the standard deviation was determined to be 0.56 µm for the particles in Figure 4.28.

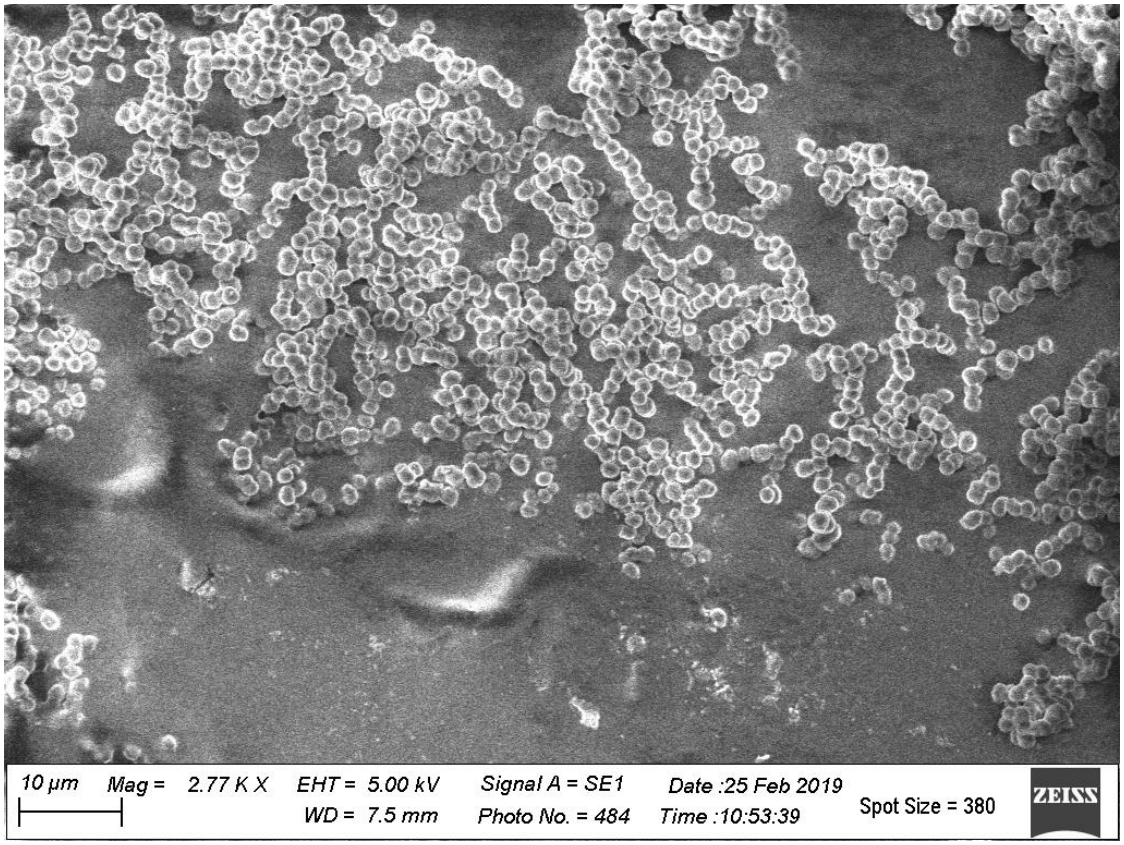


Figure 4.29: SEM micrograph of a non-size fractionated non-imprinted polymer (NIP) sample on a conductive carbon tab

The micrograph displayed in Figure 4.29 is also from a non-size fractionated MIP but does not appear to have the same conglomerates as shown in Figure 4.28. The average size for the particles in Figure 4.29 was determined to be 2.04 µm (N=30) with a standard deviation of 0.25 µm.

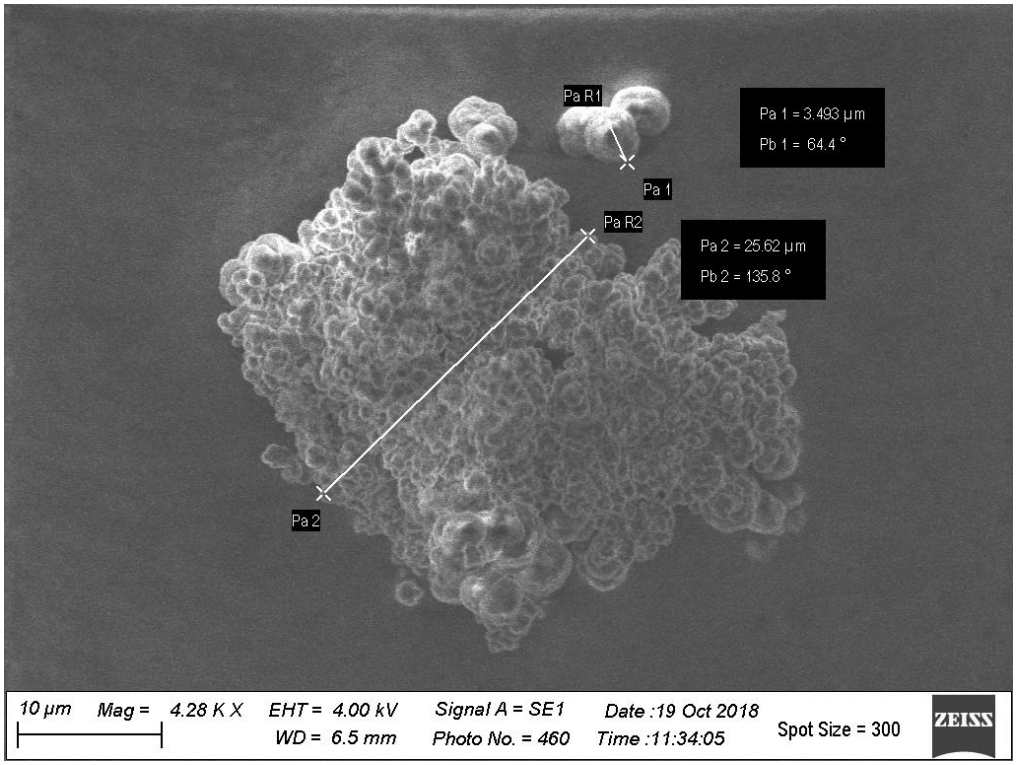


Figure 4.30: SEM micrograph of a size fractionated (24 to 52 µm) non imprinted polymer (NIP) mounted on a conductive carbon tab

Figure 4.30 shows a NIP after size fraction (Section 3.5.1), where the sieves used had apertures of 52 and 24 µm, respectively. The average diameter of the beads was determined to be 2.96 µm with a standard deviation of 0.43 µm. There appears to be smaller beads or fractions that have broken off the main conglomerate. This may happen during SPE packing thus these smaller particles could pass through the SPE frit which has a pore size of 20 µm. The origin of these small particles is not fully understood; since they were present after the polymer was wet sieved, they must have formed after the sieving procedure. It should be noted that after the sieving there appears to be much less of these loose unconglomerated particles.

Figure 4.31 and 4.32 depict the same SEM micrograph of size fractionated (24 to 52 µm) atrazine MIPs before template removal with different measurements of the particles indicated on the microgram. The average diameter for the beads was determined to be 2.03 µm with a standard deviation of 0.27 µm. The micrograph displayed in Figure 4.33 depicts size fractionated atrazine MIP from the same batch after template removal. The average diameter of the beads was determined to be 2.06 µm with a standard deviation of 0.31 µm.

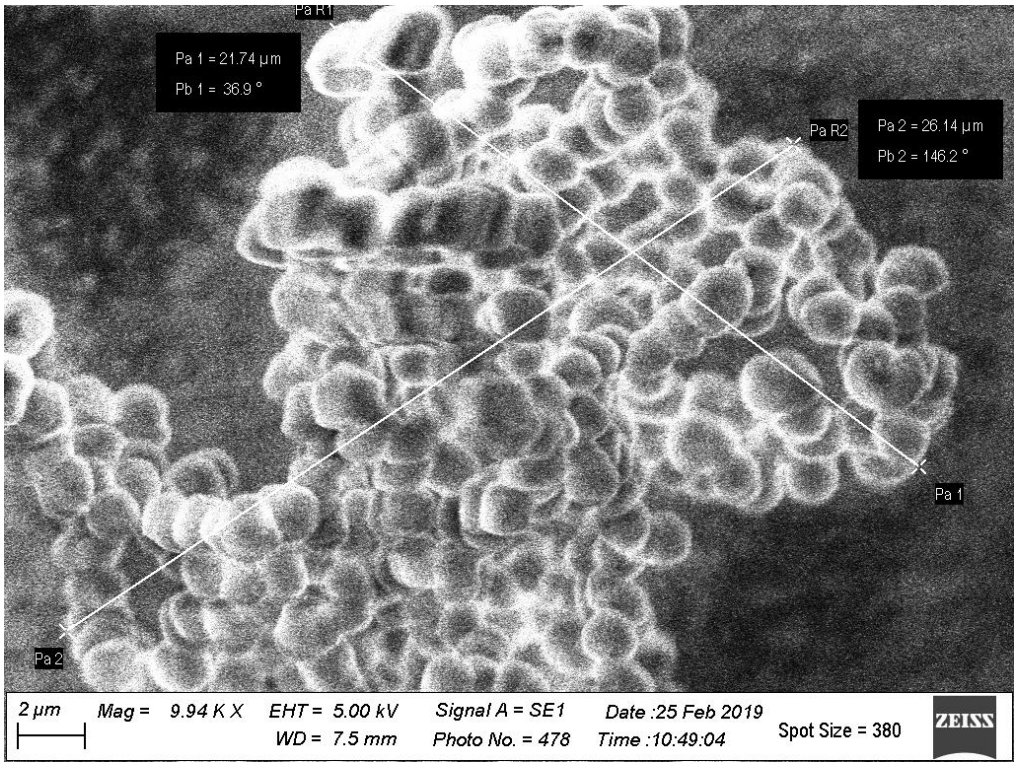


Figure 4.31: SEM micrograph of size fractionated atrazine MIP (24 µm < MIP < 52 µm) which shows the conglomerate to be 26 µm across

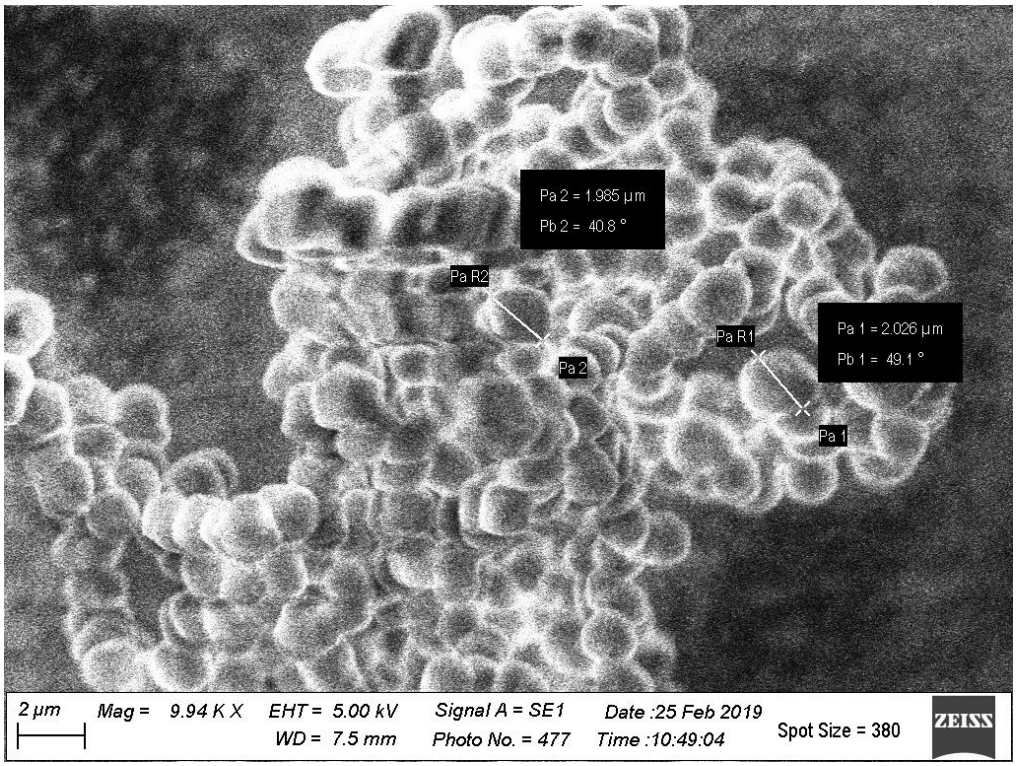


Figure 4.32: SEM micrograph of size fractionated atrazine MIP (24 µm < MIP < 52 µm) before template removal, with the bead size about 2 µm in diameter

Figure 4.32 illustrates the “unwashed” MIP, where the atrazine template has not yet been removed. Figure 4.33 is from the atrazine MIP after template removal, it has been washed with a methanol and acetic acid (9:1) solution seven times and then dried before mounting on the conductive carbon tab.

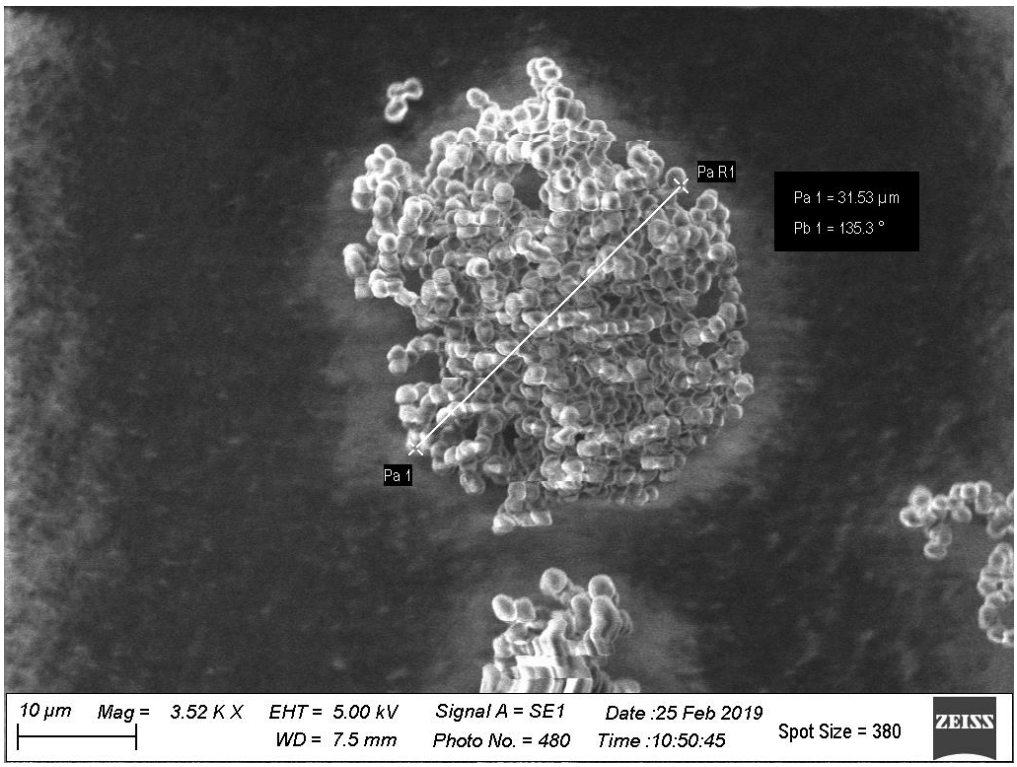


Figure 4.33: Washed size fractionated atrazine MIP (24 µm < MIP < 52 µm)

When comparing Figure 4.32 with Figure 4.33, of the washed and unwashed atrazine MIP, there appears to be little to no morphological differences in the bead shape or size. This indicates that the removal of the template or the washing process did not disturb the morphology of the atrazine MIP. Since the whole MIP principle depends on the rigidness of the molecular cavity after the template has been removed, these two images were very significant in demonstrating that the synthesis was effective in this regard.

The surface area and volume of a single bead

Although the beads were not observed to be 100% spherical, this was assumed for calculation purposes. The following formula was used to calculate the volume of a sphere:

$$V = \frac{4}{3}\pi r^3$$

Where *V* represents the volume of the sphere, and *r* the radius. For a particle with a 2 µm diameter, the radius is 1 µm, the volume thus calculates to 4.19 μm^3.

The area of a sphere was calculated using the formula:

$$A = 4\pi r^2$$

Where *A* represents the surface area. For the same particle, the area was calculated to 12.57 μm^2.

The surface area to volume ratio is given by:

$$\frac{A}{V} = \frac{3}{r}$$

Thus, for the particle with a 2 μm diameter, the area to volume ratio is 3.

Comparison to literature

A similar dummy template chloroacetamide MIP, with MAA as functional monomer and EDGMA as crosslinker but with acetonitrile as porogen, was characterized in a similar manner as reported in literature (Wang *et al.*, 2015). The bead sizes and shapes were remarkably similar to that obtained in this study at approximately 2 μm diameter.

4.4.2: FTIR Spectra

The FTIR spectra were used to compare the presence and relative abundance of different functional groups and chemical bonds in the various MIPs.

Table 4.34 contains peaks that were commonly found in synthesised polymer FTIR spectra and their associated chemical bonds, as was the case for the NIP FTIR spectrum (Figure 4.34).

Table 4.34: Commonly observed FTIR peaks for the synthesised polymers (as reported in Jeffery *et al.,* 1903)

Approximate peak location (cm^{-1})	**Peak Shape**	**Associated functional group/ bond**
3300	Broad and flat	O-H
2990	Low %T and no baseline separation	Asymmetric 1°C-H
2950	Low %T and no baseline separation	Symmetric 1°C-H
1720	Sharp	C=O
1640	Shoulder peak	C=C
1450	Split peak	Asymmetric 2°C-H
1390	Sharp	Symmetric 2°C-H
1255	Sharp	Asymmetric C–O–C
1150	Sharp -Largest peak	Symmetric C–O–C
1125	Low %T- not visible in all	Carboxylic groups
>1000	Present, but not always distinguishable	Vinylic C-H

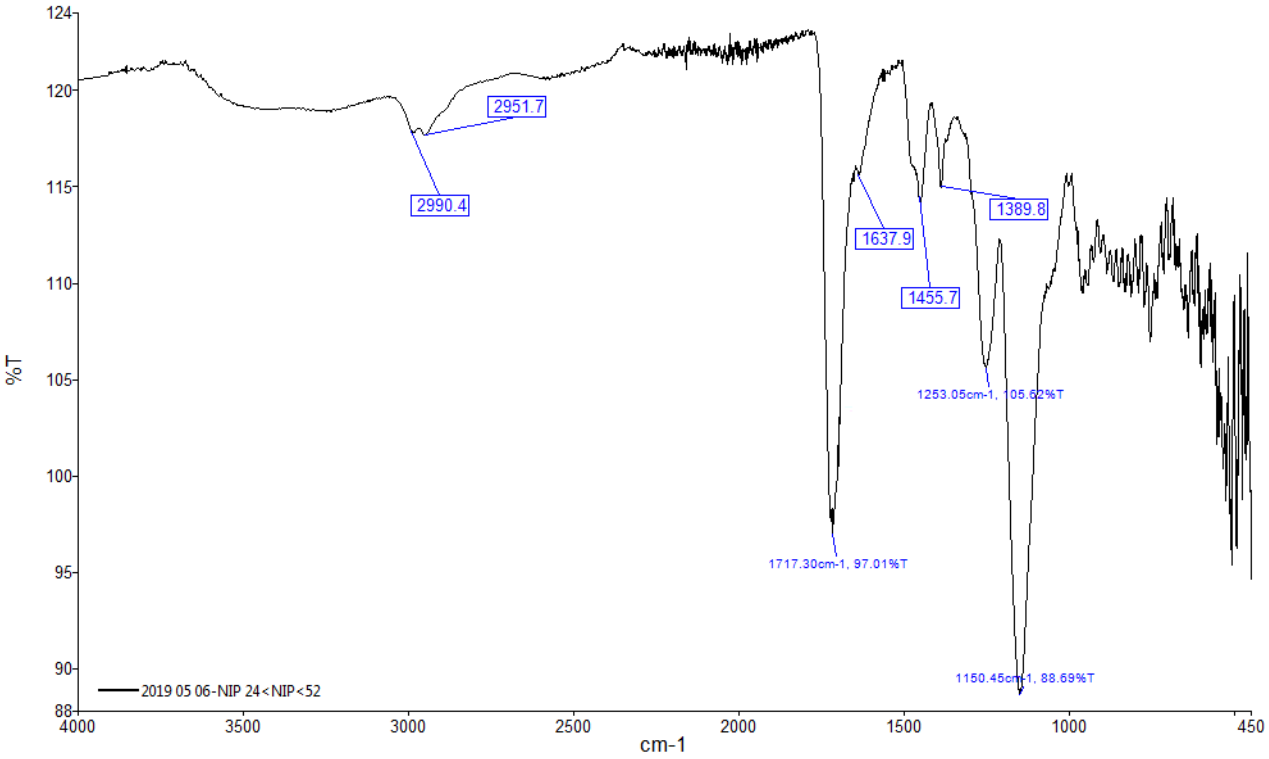


Figure 4.34: NIP FTIR spectrum

For the NIP FTIR spectrum (Figure 4.34), the broad peak in the region of 3300 cm^{-1} is associated with O-H stretching. Peaks in the region of 2990 and 2950 cm^{-1} were assigned to the asymmetric and symmetric stretching vibrations of the C–H in methyl groups or primary carbons. The strong peak in the region of 1720 cm^{-1} can be assigned to C=O bonds. The very small shoulder peak is in the region typically associated with C=C stretching vibrations. The smaller peaks at 1450 and 1389 cm^{-1} were associated with the asymmetric and symmetric vibrations of C–H of methylene groups or secondary carbons. The peaks at 1255 and 1150 cm^{-1} could be associated with C–O–C asymmetric and symmetric stretching vibrations of the ether group. The strongest peak, in the region of 1125 cm^{-1}, can possibly be attributed to carboxylic groups. The small peaks in the region below 1000 cm^{-1} are associated with the C-H bonds of vinylic carbons (Jeffery *et al.*, 1903). Refer to Section 2.7.2.2, where the polymerization mechanism was discussed, for the chemical structures of the monomers and subsequent polymer. The C=O bonds are present in both MAA and EDGMA and explain the sharp peak in the FTIR spectrum. The largest peak, representing C–O–C at 1150 cm^{-1}, also relates to the chemical structure of EDGMA that contains two ester functional groups.

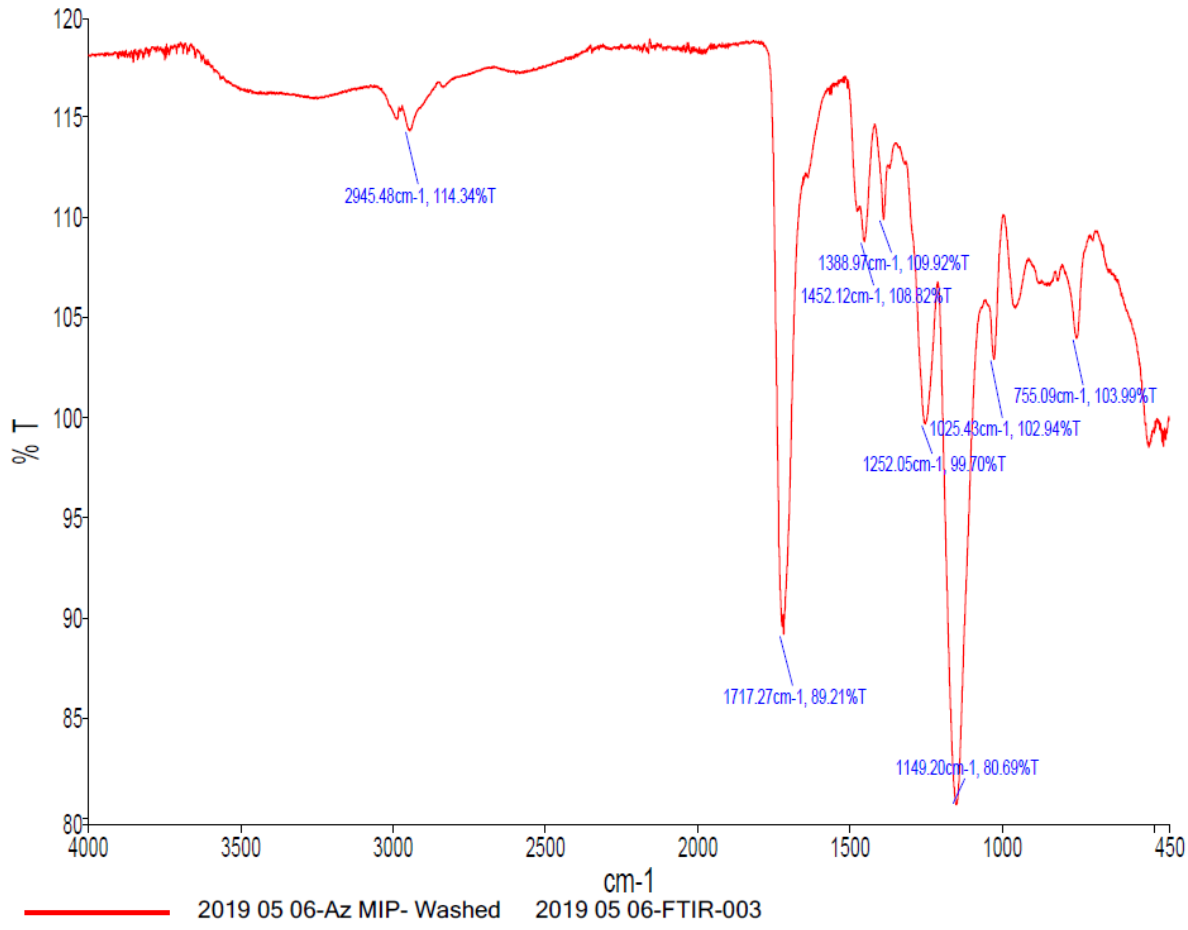


Figure 4.35: FTIR spectrum of the washed atrazine MIP

The atrazine MIP FTIR spectrum (Figure 4.35) has very similar peaks compared to the NIP. The strongest bands were almost identical, whilst the largest difference was the peak at 1025 cm^{-1} and the region of < 1000 cm^{-1} was observed to be better defined. The 1025 cm^{-1} peak represents carboxylic groups, and as discussed in Section 2.7.2.3, atrazine does have a major influence on the orientation of the MAA functional monomer orientation because of hydrogen bonding with the carboxylic group. It can be theorised that the carboxylic groups were more exposed or orientated on the surface of the atrazine MIP and were thus more easily detectable by the FTIR resulting in a more resolved peak than when compared to the NIP spectrum.

Figure 4.36 compares the FTIR spectra of the atrazine MIP with and without template. The template was removed with a 9:1 methanol: acetic acid mixture for the washed MIP. The compare function on the FTIR software reported a 98% correlation between the two spectra.

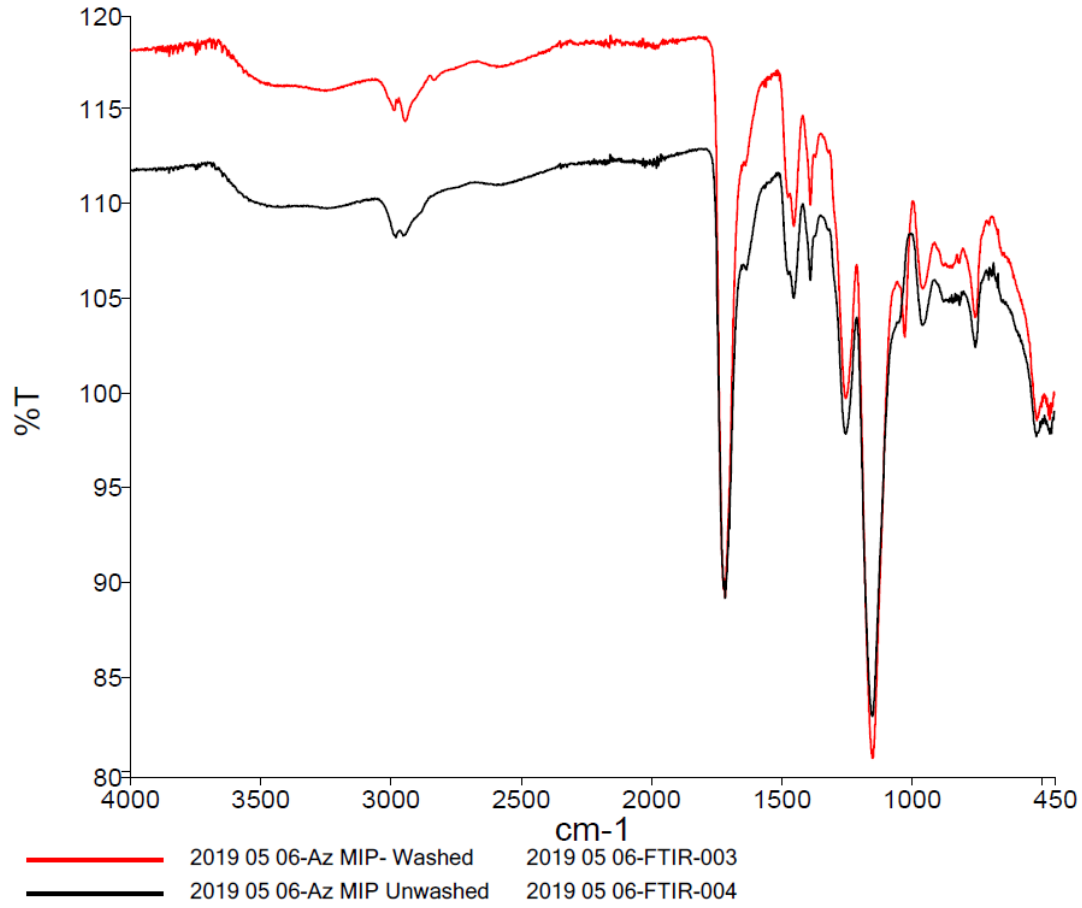


Figure 4.36: Atrazine MIP FTIR spectra, where the red spectrum represents the washed MIP and the black spectrum represents unwashed MIP. There does not appear to be any notable differences between the two spectra

A literature FTIR spectrum for atrazine (Figure 4.37) revealed a large peak at a wavenumber of 1578 cm^{-1}. There was thus not enough atrazine in the unwashed MIP relative to the amount of polymer present to leave a detectable peak in the black FTIR spectrum (Figure 4.36).

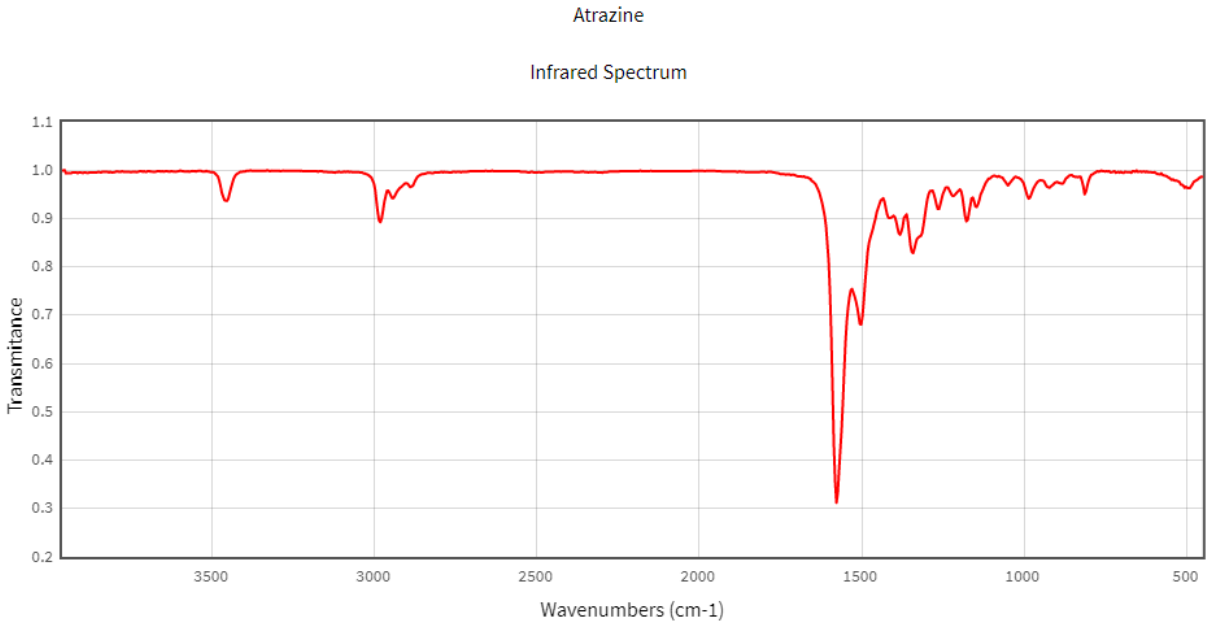


Figure 4.37: Atrazine infrared spectrum from the NIST library. The largest transmittance wavenumber was reported to be 1578 cm^{-1} (NIST, 2014)

Figure 4.38 depicts the FTIR spectrum for an unwashed terbuthylazine MIP.

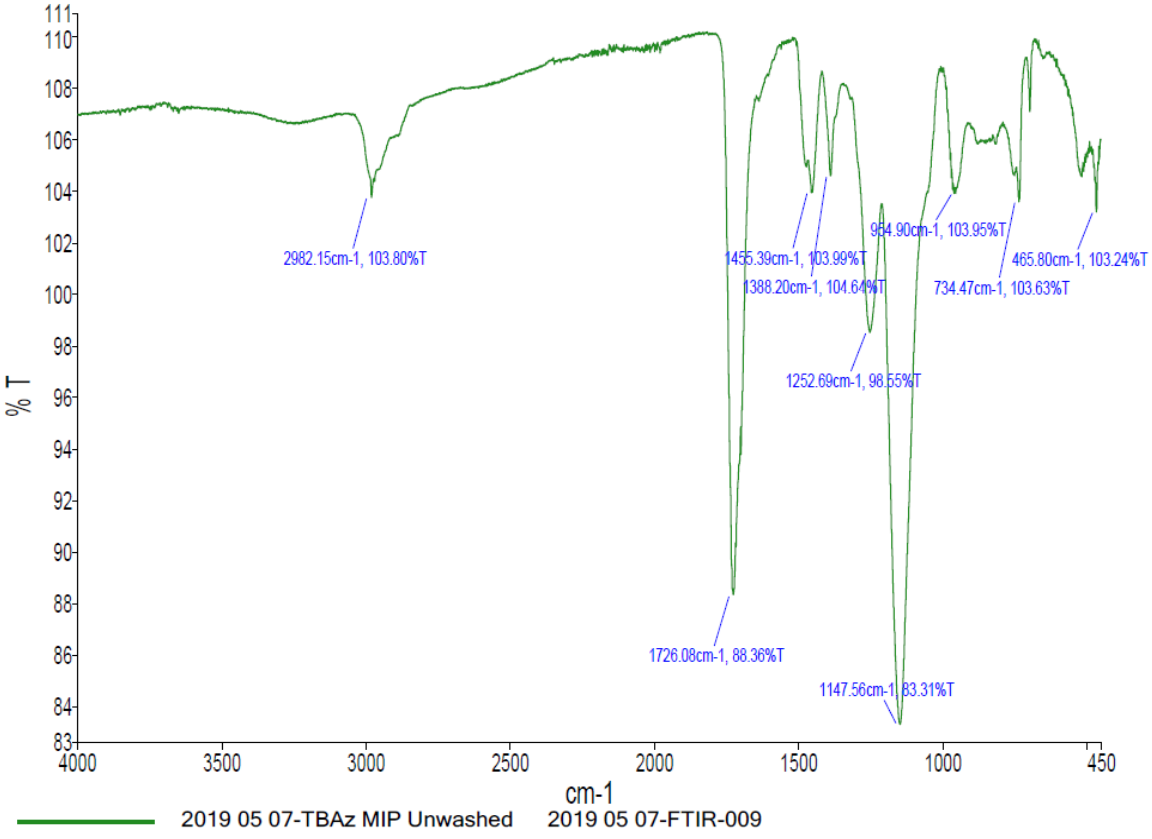


Figure 4.38: Terbuthylazine MIP FTIR spectrum

The terbuthylazine MIP spectrum (Figure 4.38) was once again comparable to the NIP and atrazine MIP spectra, the biggest difference was observed to be the peak at 2982 cm^{-1}, that possibly represents asymmetric 1°C-H bonds, which was more defined. This indicates asymmetric primary carbons are possibly more prevalent than symmetric primary carbons in the terbuthylazine MIP when compared to the atrazine MIP. This may be due to the chemical structure of terbuthylazine, as it contains four primary carbons where atrazine only contains three.

Figure 4.39 and 4.40 depict the FTIR spectra of acetochlor and alachlor MIPs, respectively.

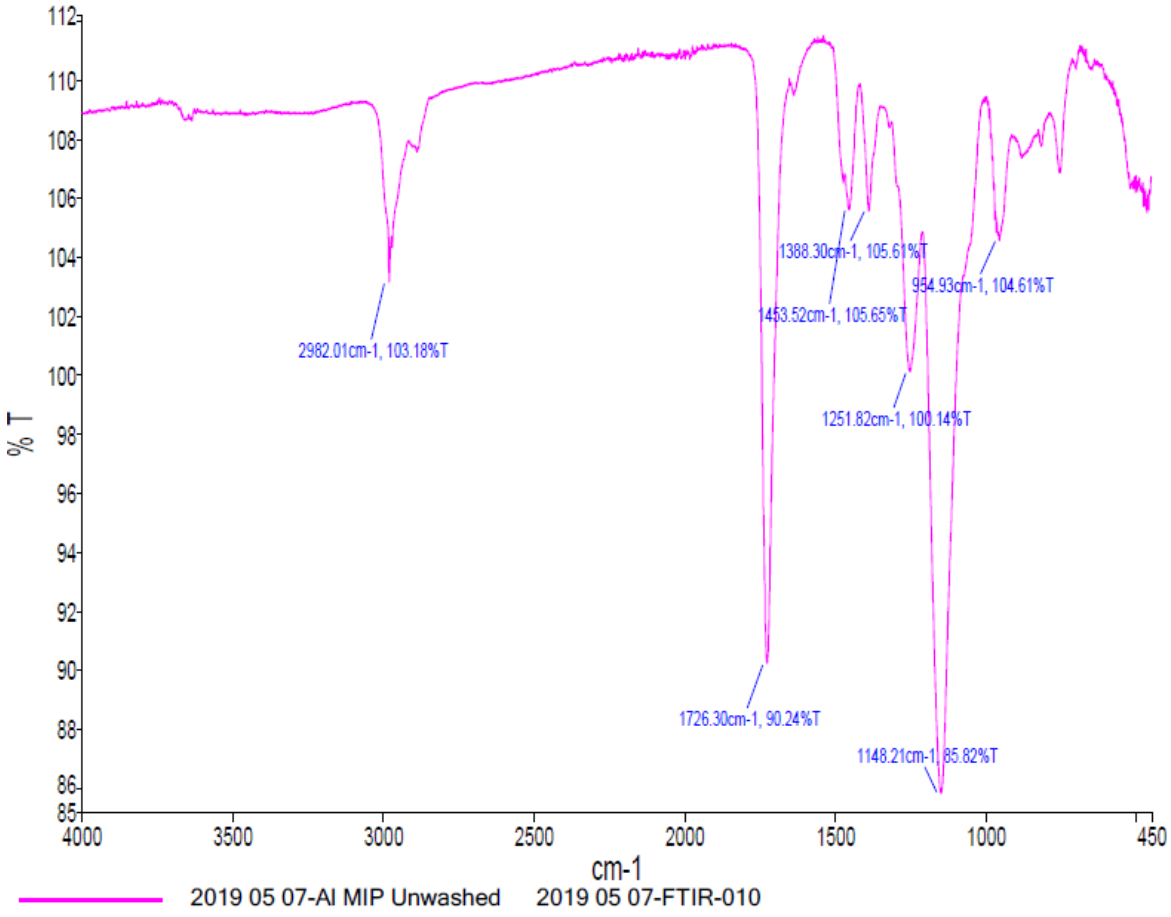


Figure 4.39: Alachlor MIP FTIR spectrum

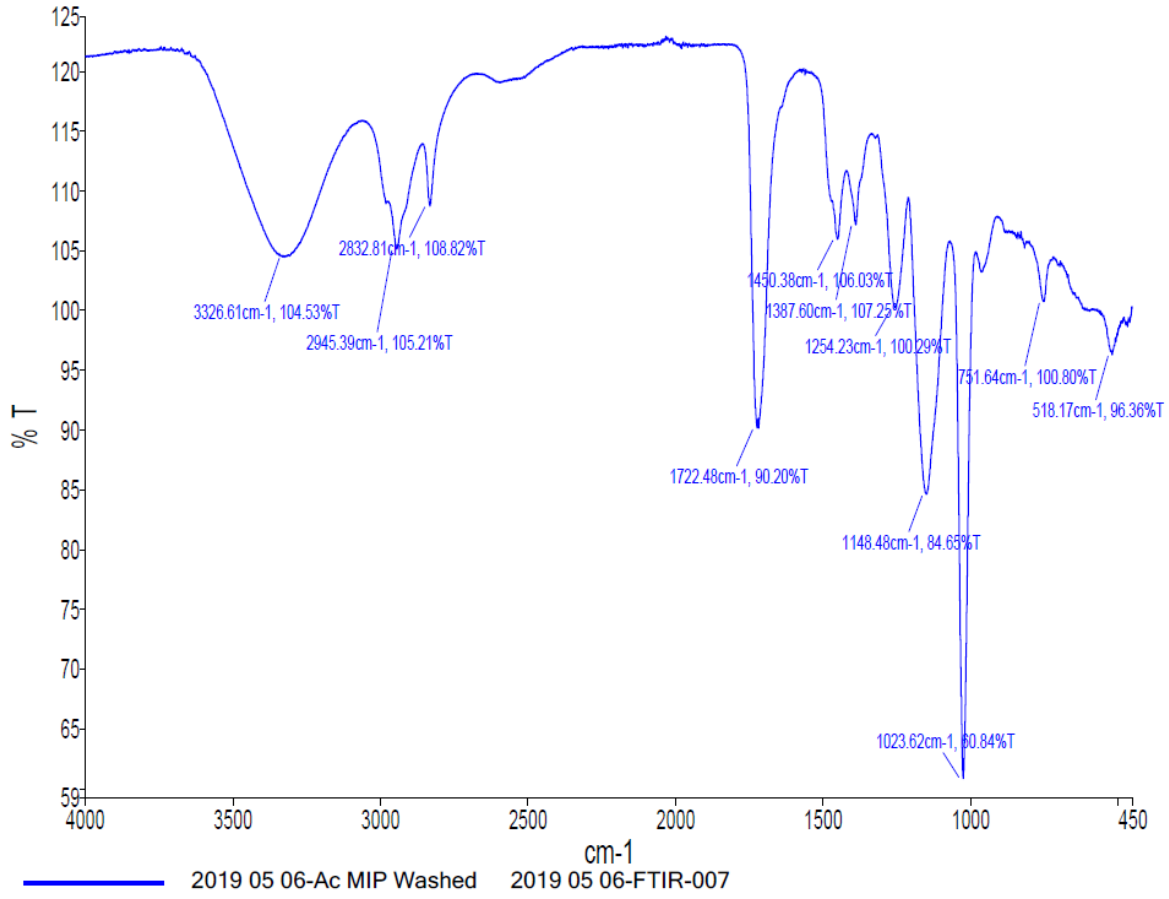


Figure 4.40: Acetochlor MIP FTIR spectrum

With reference to Figure 4.40, the broad peak in the region of 3320 cm^{-1} can be associated with O-H stretching, and was reported to be the most sensitive to the template removal (Wang *et al.,* 2015). It is interesting to note that the O-H peak was most defined in the washed acetochlor MIP (Figure 4.40). From Section 2.7.2.2, where the polymerization mechanism was discussed and all the chemical structures are displayed, the only O-H bond that was present is the carboxylic group of MAA.

For both the FTIR spectra of alachlor and acetochlor MIPs (Figure 4.39 and Figure 4.40) the peaks in the region of 2830 and 2945 cm^{-1} were assigned to the asymmetric and symmetric stretching vibrations of the C–H in methyl groups or primary carbons. The strong peak in the region of 1720 cm^{-1} can be assigned to C=O bonds. The smaller peaks at 1450 and 1387 cm^{-1} are associated with the asymmetric and symmetric vibrations of C–H of methylene groups or secondary carbons. The peaks at 1255 and 1150 cm^{-1} are associated with C–O–C asymmetric and symmetric stretching vibrations of the ether group. The strongest peak, in the region of 1020 cm^{-1} can possibly be attributed to carboxylic groups. The small peaks at in the region below 1000 cm^{-1} are associated with the C-H bonds of vinylic carbons.

Similar FTIR spectra were reported in the literature, when the same monomers and activator were used and butachlor (a chloroacetanilide pesticide) was the template molecule (Wang *et al.,* 2015).

4.5: Preliminary SPE results

This section presents results for both the MISPE and C18 SPE methods prior to method optimization. These experiments were done under ideal conditions, wherein relatively high concentrations of analytes were extracted and only water was used to wash the sorbent before elution, as was discussed in Section 3.6. The aim of these experiments was to demonstrate how the MISPEs function under ideal conditions.

4.5.1: C18 SPE

4.5.1.1: C18 SPE experiment with a higher concentration of atrazine, acetochlor, terbuthylazine and alachlor

All the fractions (load, wash and elution) were collected and analysed. The relevant peaks were integrated and the results are presented in Table 4.35. The load and wash fractions contained no traces of the pesticides demonstrating the C18 sorbent adsorbs the pesticide effectively from aqueous solutions. Most of the pesticide was collected in the first 2 mL of the methanol elution fraction. All the pesticides had a smaller peak area and subsequent calculated concentration than that of the control sample, and terbuthylazine was the most affected analyte with the lowest recovery. As discussed in Section 3.6.1.a, an injection split of 3:1 was used to ensure the concentration was in the calibration range, and a multiplier of 3 was added in the software to mathematically correct for this. The control sample was made up at 50 µg/mL with 5 µg/mL caffeine and injected with the same spilt and multiplier to confirm the calibration curves were still valid. It should be noted that much more in-depth recovery experiments were done in Section 4.7 at relevant concentrations.

Table 4.35: Summary of all the collected fraction peak areas for each pesticide extracted using a C18 SPE from a 3 mL spiked water sample at 33 µg/mL

Pesticide	Atrazine		Terbuthylazine		Acetochlor		Alachlor	
	µg/mL	Recovery (%)	µg/mL	Recovery (%)	µg/mL	Recovery (%)	µg/mL	Recovery (%)
Control	50.5	101	52.1	104.2	49.3	98.6	48.9	97.8
Load-(Water)	ND	ND	ND	ND	ND	ND	ND	ND
Wash-(Water)	ND	ND	ND	ND	ND	ND	ND	ND
Elution 1-(MeOH)	47.4	94.8	43.2	86.4	47.1	94.2	49	98
Elution 2-(MeOH)	0.1	0.1	0.2	0.4	0.06	0.1	0.05	0.1

Pesticide	Atrazine		Terbuthylazine		Acetochlor		Alachlor	
	µg/mL	Recovery (%)	µg/mL	Recovery (%)	µg/mL	Recovery (%)	µg/mL	Recovery (%)
Elution 3- (MeOH)	ND	ND	ND	ND	ND	ND	ND	ND

From Table 4.35 it was found that the 3:1 split and subsequent multiplier worked effectively, as the control sample was observed to be approximately 50 µg/mL for each analyte.

The C18 SPE performed well in terms of recovery, and matrix effects appeared to be minimal at this concentration level of 33 µg/mL. The elution efficiency was also excellent, as there was very little of any pesticide eluting in the second fraction and no pesticide was detected in the third fraction, indicating only 2 mL of methanol was required to elute the pesticides from the C18 sorbent.

4.5.1.2: C18 SPE experiment for the cannabis matrix at lower atrazine concentration

Table 4.36 displays the results obtained from the spiked cannabis samples; the methodology was discussed in Section 3.6.1.b. This experiment was done at 0.05 mg/g concentration (atrazine/ plant material) on the cannabis flower, still far above the 0.05 mg/kg MRL (in Section 4.7 the relevant concentration was subsequently analysed). Five repeats were performed for statistical analysis. A multiplier of 2 was added to the run list, to compensate for only taking 1 mL of the 2 mL elution fraction. A dilution factor of 0.05 was also added to the run list to compensate for the evaporation of 1 mL eluent and reconstitution with 50 µL methanol containing internal standard. The calculated concentration in the 1 mL elution fraction was 0.5 µg/mL. After the reconstitution, the concentration was calculated to be 10 µg/mL, right in the middle of the calibration curve. A control sample was prepared at this same concentration and also evaporated and analysed with the samples to verify the calibration curve was still valid and the correct multipliers were used.

Table 4.36: Summary of the peak areas in the control and cannabis samples

Sample		Atrazine concentration (µg/mL) in elution fraction	Calculated atrazine concentration on flower (µg/mg)	Recovery (%)
Control		0.51	N/A	102.0
Cannabis matrix elution fractions repeats	1	0.45	0.045	90.6
	2	0.48	0.048	96.0
	3	0.47	0.047	93.8
	4	0.44	0.044	88.0
	5	0.52	0.052	104.0
Average recovery		**0.47**	**0.047**	**94.5**
Standard deviation		0.0274	0.00274	5.49
			RSD (%)	5.81

Since 20 mg plant material was diluted into 2 mL water, the final concentration of atrazine on the flower could be back calculated.

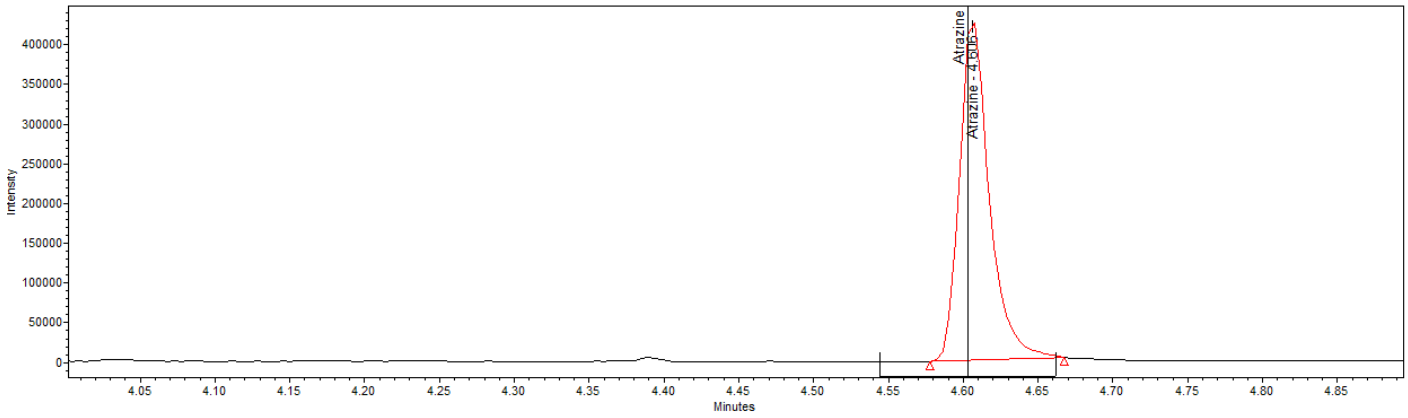


Figure 4.41: The atrazine (m/z 200 Da) chromatogram obtained from the control sample at a concentration of 10 µg/mL after evaporation and reconstitution with caffeine internal standard solution

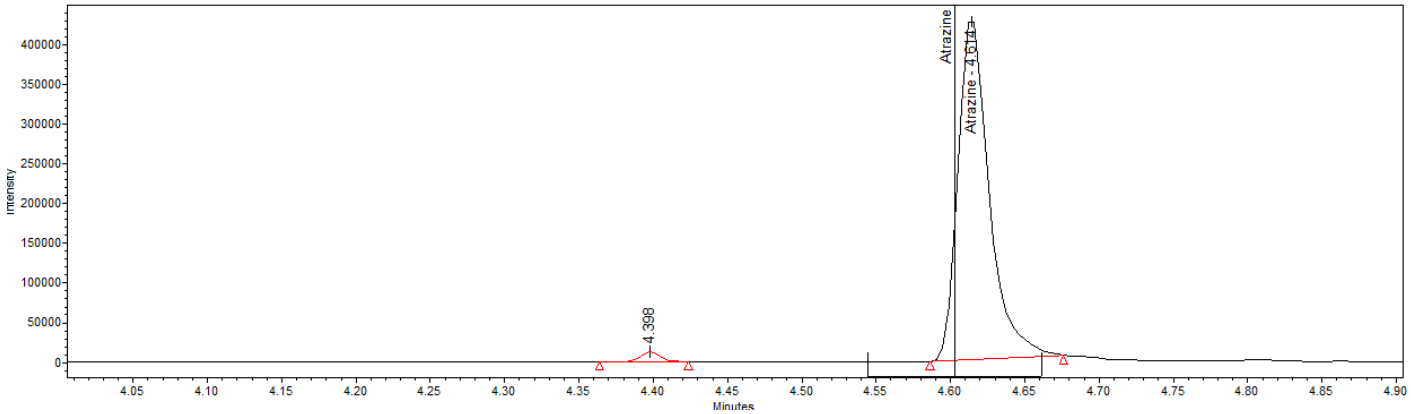


Figure 4.42: The atrazine (m/z 200 Da) chromatogram obtained from the spiked cannabis (0.05 µg/mg) elution fraction following C18 SPE and subsequent vacuum concentration

After the elution fractions were dried utilising vacuum concentration, an IS caffeine solution was used to reconstitute the samples to a constant volume with a known concentration of internal standard. The IS was thus only used for the instrumental method post SPE.

4.5.2: Comparison of NISPE and MISPE

The following sections are divided into each target pesticide and their respective synthesised MIPs and subsequent MISPE and NISPE results. The methodology was discussed in Section 3.6.2. These experiments were performed by spiking 3 mL of MilliQ water to a final concentration of 0.208 µg/mL. All the fractions were dried via vacuum concentration and reconstituted with 50 µL of 5 µg/mL caffeine in methanol giving a total volume of 50 µL. The theoretical concentration was calculated to be 12.5

µg/mL for the pesticides. These experiments were done under ideal conditions, where the sorbent was washed with only water after the loading fraction was passed through the sorbent. Since only water was used as matrix in the loading fraction, any interferences or contaminants present would have come from the sorbent. Two 1.5 mL methanol fractions were used to elute the pesticides from the sorbent.

To ensure the adsorption capacity of the sorbent was sufficient to completely trap the target analytes the following calculation was performed for 100 mg of polymer packed into a SPE cartridge:

$$Adsorption\ capacity\ (mg/g) = \frac{(C_0 - C_e)V}{W}$$

Where the following values are known:

W = 100 mg or 0.1 g

V = 3 mL or 0.003 L

C_0 = (0.025 mL x 25 µg/mL) /3 mL = 0.208 µg/mL or 0.208 mg/L

C_e = 0 mg/L

$$Adsorption\ capacity\ (mg/g) = \frac{(0.208 - 0)0.003}{0.1} = 0.006\ mg/g$$

In Section 4.3, it was found that all the synthesised MIPs and NIPs have a far greater adsorption capacity (>0.5 mg/g), thus theoretically all the synthesised polymers have sufficient capacity to adsorb all the pesticides at the given concentrations.

It was observed that almost all the water loading and wash fractions contained small amounts of pesticide, well below the range of the calibration curve which had the lowest point at 2.5 µg/mL. Analyte bleeding can likely be subscribed to channelling in the packing of the MISPE/NISPE sorbent.

4.5.2.1: Atrazine recovery from NISPE and atrazine MISPEs

Prior to the use of the atrazine MISPE sorbent, methanol was passed through the sorbent bed, both to condition the packing and to ensure all the atrazine template had been removed.

Table 4.37, 4.38 and 4.39 represent the atrazine recovery for the NISPE, MISPE and EAC MISPE respectively.

Table 4.37: NISPE atrazine recovery with two separate elution fractions

Fraction	Atrazine concentration (µg/mL)	Atrazine recovery (%)
Milli Q Loading fraction (3 mL)	0.07	0.60
Milli Q Wash fraction (3 mL)	0.03	0.21
1st Methanol elution fraction (1.5 mL)	6.31	50.50
2nd Methanol elution fraction (1.5 mL)	5.75	45.98
Total recovery	12.16	97.29

Table 4.38: MISPE atrazine recovery with two separate elution fractions

Fraction	Atrazine concentration (µg/mL)	Atrazine recovery (%)
Milli Q Loading fraction (3 mL)	0.04	0.32
Milli Q Wash fraction (3 mL)	0.01	0.12
1st Methanol elution fraction (1.5 mL)	6.42	51.39
2nd Methanol elution fraction (1.5 mL)	5.40	43.20
Total recovery	11.88	95.02

Table 4.39: EAC MISPE atrazine recovery with two separate elution fractions

Fraction	Atrazine concentration (µg/mL)	Atrazine recovery (%)
Milli Q Loading fraction (3 mL)	0.03	0.21
Milli Q Wash fraction (3 mL)	0.01	0.12
1st Methanol elution fraction (1.5 mL)	8.93	71.46
2nd Methanol elution fraction (1.5 mL)	3.33	26.66
Total recovery	12.31	98.45

From Table 4.37, 4.38 and 4.39 it was clear that the total recovery for atrazine from each sorbent was very high under these ideal experimental conditions. Figure 4.43 displays an example chromatogram of the atrazine peak after elution from the EAC MISPE cartridge.

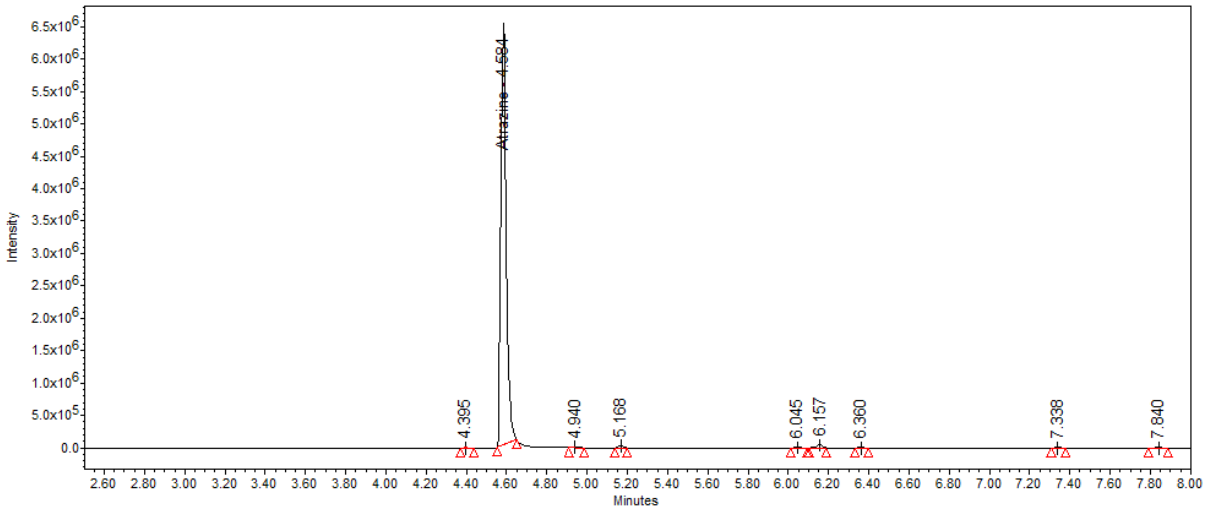


Figure 4.43: An example SIR chromatogram from atrazine (m/z 200 Da) as obtained from the 1st methanol elution fraction after the atrazine EAC MISPE procedure

When compared to the CI8 SPE, the elution efficiency was decreased on the atrazine MIP, as the second elution fraction contained a significant amount of atrazine. This was likely because of the drying step that was omitted from the MISPE method (Figure 3.1) to prevent cracking and channelling of the synthesised polymer bed when it dried. It was also observed that very little atrazine was passed through the MISPE in the aqueous loading fractions. It might also be theorized that the MIP sorbent adsorbs the atrazine analyte more strongly than the C18 sorbent.

The atrazine recoveries obtained in the above experiment are comparable to those reported in literature. A recovery range of 94 to 99% was reported for atrazine on a similar atrazine MIP (Kueseng *et al.*, 2009) whilst a recovery of 74.6% was reported for atrazine on a similar MIP, but with DCM as wash solvent and 20.2% of the atrazine was reported in the wash solvent (Guzzella *et al.*, 2008).

4.5.2.2: Terbuthylazine recovery from NISPE and terbuthylazine MISPEs

Table 4.40, 4.41 and 4.42 display recoveries obtained for terbuthylazine on a NISPE and variations of terbuthylazine MIPs on self-packed cartridges.

Table 4.40: NISPE terbuthylazine recovery with two separate elution fractions

Fraction	Terbuthylazine concentration (µg/mL)	Terbuthylazine recovery (%)
Milli Q Loading fraction (3 mL)	0.04	0.36
Milli Q Wash fraction (3 mL)	0.01	0.07
1st Methanol elution fraction (1.5 mL)	9.30	74.43
2nd Methanol elution fraction (1.5 mL)	2.26	18.04
Total recovery	11.61	92.90

Table 4.41: MISPE terbuthylazine recovery with two separate elution fractions

Fraction	Terbuthylazine concentration (µg/mL)	Terbuthylazine recovery (%)
Milli Q Loading fraction (3 mL)	0.04	0.33
Milli Q Wash fraction (3 mL)	0.01	0.03
1st Methanol elution fraction (1.5 mL)	9.62	76.95
2nd Methanol elution fraction (1.5 mL)	1.91	15.26
Total recovery	11.57	92.57

Table 4.42: EAC MISPE terbuthylazine recovery with two separate elution fractions

Fraction	Terbuthylazine concentration (µg/mL)	Terbuthylazine recovery (%)
Milli Q Loading fraction (3 mL)	0.04	0.03
Milli Q Wash fraction (3 mL)	0.03	0.03
1st Methanol elution fraction (1.5 mL)	8.97	71.77
2nd Methanol elution fraction (1.5 mL)	2.31	18.45
Total recovery	11.29	90.28

As with atrazine, the elution efficiency of the terbuthylazine MIP also differs when compared to the C18 results in Section 4.5.1, as there was observed to be a significant concentration of terbuthylazine in the second 1.5 mL elution fractions for all the self-packed cartridges. It was possibly because the drying step was omitted, thus there was still some water left in the sorbent bed that dilutes the methanol eluant when it was passed through the sorbent bed.

In literature, a much lower recovery of 68.5% was reported for terbuthylazine on a similar MIP. DCM was used as wash solvent and a loss of 21.3% was reported during the washing step (Guzzella *et al.*, 2008).

4.5.2.3: Acetochlor recovery from NISPE and acetochlor MISPEs

Recovery results for alachlor on self-manufactured NISPE and alachlor MISPEs cartridges are presented in Table 4.43, 4.44 and 4.45.

Table 4.43: NISPE acetochlor recovery with two separate elution fractions

Fraction	Acetochlor concentration (µg/mL)	Acetochlor recovery (%)
Milli Q Loading fraction (3 mL)	0.14	1.11
Milli Q Wash fraction (3 mL)	0.19	1.56
1st Methanol elution fraction (1.5 mL)	9.30	74.40
2nd Methanol elution fraction (1.5 mL)	1.38	11.04
Total recovery	**11.01**	**88.11**

Table 4.44: MISPE acetochlor recovery with two separate elution fractions

Fraction	Acetochlor concentration (µg/mL)	Acetochlor recovery (%)
Milli Q Loading fraction (3 mL)	0.08	0.67
Milli Q Wash fraction (3 mL)	0.19	1.52
1st Methanol elution fraction (1.5 mL)	9.75	78.00
2nd Methanol elution fraction (1.5 mL)	1.89	15.09
Total recovery	**11.91**	**95.29**

Table 4.45: EAC MISPE acetochlor recovery with two separate elution fractions

Fraction	Acetochlor concentration (µg/mL)	Acetochlor recovery (%)
Milli Q Loading fraction (3 mL)	0.08	0.66
Milli Q Wash fraction (3 mL)	0.14	1.11
1st Methanol elution fraction (1.5 mL)	8.75	69.98
2nd Methanol elution fraction (1.5 mL)	2.53	20.24
Total recovery	**11.50**	**92.00**

The same trend was observed as with the previous MIPs, in terms of the reduced elution efficiency on the MISPEs when compared to the C18 SPE experiments from Section 4.5.1, as there were significant amounts of acetochlor in the second 1.5 mL methanol elution fraction.

The acetochlor recoveries found in this experiment are slightly higher than the 80.6 – 90.2% reported for a similar MIP (Wang *et al.*, 2015). This was probably because no real wash solvent has yet been implemented, whilst Wang *et al.* employed hexane as a wash solvent.

4.5.2.4: Alachlor recovery from NISPE and alachlor MISPEs

Table 4.46, 4.47 and 4.48 display the NISPE and alachlor MISPE results obtained for alachlor with loading, wash and elution fractions which were dried and reconstituted with methanol containing the internal standard. The methodology was discussed in Section 3.6.2.

Table 4.46: NISPE alachlor recovery with two separate elution fractions

Fraction	Alachlor concentration (µg/mL)	Alachlor recovery (%)
Milli Q Loading fraction (3 mL)	0.23	1.82
Milli Q Wash fraction (3 mL)	0.11	0.86
1st Methanol elution fraction (1.5 mL)	7.82	62.60
2nd Methanol elution fraction (1.5 mL)	3.36	26.88
Total recovery	**11.52**	**92.15**

Table 4.47: MISPE alachlor recovery with two separate elution fractions

Fraction	Alachlor concentration (µg/mL)	Alachlor recovery (%)
Milli Q Loading fraction (3 mL)	0.11	0.86
Milli Q Wash fraction (3 mL)	0.19	1.51
1st Methanol elution fraction (1.5 mL)	7.89	63.14
2nd Methanol elution fraction (1.5 mL)	3.73	29.86
Total recovery	**11.92**	**95.37**

Table 4.48: EAC MISPE alachlor recovery with two separate elution fractions

Fraction	Alachlor concentration (µg/mL)	Alachlor recovery (%)
Milli Q Loading fraction (3 mL)	0.09	0.74
Milli Q Wash fraction (3 mL)	0.11	0.86
1st Methanol elution fraction (1.5 mL)	9.84	78.72
2nd Methanol elution fraction (1.5 mL)	1.91	15.28
Total recovery	**11.95**	**95.60**

The same observed trend persists thought all the MIPs, in that the elution efficiency was adversely affected by the absence of the drying step after the SPE loading and wash fractions were passed through the cartridge. The C18 SPE has excellent elution efficiency because pure methanol can be passed through the sorbent for the elution fraction.

The alachlor recoveries obtained in this study are higher than the 80.6 – 90.2% range reported for a similar MIP (Wang *et al.*, 2015). This was probably because no wash solvent has yet been implemented. For the literature example referred to earlier, were a Wang *et al*. employed hexane as a wash solvent and methanol as the elution solvent.

4.5.3: Experimental difficulties and observations made during preliminary SPE and MISPE experiments

There should be negligible amounts of pesticide in the loading and wash fractions after they have been passed through the SPE and it should be possible to elute the analytes with minimal solvent and subsequent concentration.

Several issues were observed regarding the synthesised polymer packed into a cartridge as compared to the commercial C18-E (100 mg) cartridge. The first problem was observed when attempting to pack the cartridges to the same height, or approximate volume, as that of the C18 cartridges. When the top frit was pushed down, the polymer compressed to a smaller volume than it first appeared. The top frit could not be removed without damaging it. Weighing approximately 175 mg of synthesised polymer was found to result in roughly the correct packing height after compression when compared to the 100 mg C18 sorbent bed. A comparative volume of MIP sorbent and C18 sorbent was thus used, instead of a weight.

The second obstacle was that the MISPE appeared to shrink when it dried. This makes the drying step after loading and washing the MISPE impractical, as this causes excessive channelling in the packed cartridge. Since the drying step was omitted from the MISPE method, the elution efficiency is reduced drastically when compared to the C18 cartridge. This was because the elution solvent must wash off the wash solvent, mostly aqueous, meaning the first fraction of elution solvent was a mixture of methanol and water. It might also be that the MISPE has a higher affinity for the template pesticide, but the increase in elution solvent was also observed for the NISPE. This was confirmed with C18 experiments in Section 4.5.1; the first elution fraction from the C18 SPE dried much faster than the same fraction from the MISPE. Drying the loading and wash fractions took a very long time in the vacuum sample concentrator because of their aqueous nature.

In conclusion of the preliminary recovery experiment, all the produced MISPEs appeared to work well under the favourable experimental conditions, where no background matrix was present, and the wash steps were performed using only water. There was minimal channelling or bleed in the aqueous loading or wash fractions. Despite not implementing the drying step, as this was observed to damage the synthesised polymer packing integrity, simply using a slightly larger volume of methanol eluted the analyte very effectively.

At the selected concentration there appeared to be little difference between the standard and the enhanced adsorption capacity MIPs.

It was concerning to find the NISPE also yielded good recoveries, with recoveries of > 90% for all four of the selected pesticides. Because the pesticides are hydrophobic, as discussed in Section 2.2.4, they are attracted to the nonpolar polymer, including the NIP. In Section 2.7.2.3 the hydrogen bonds between the monomers and pesticides were discussed. It could be argued that the NIP also contains the same monomers, and although no cavities are present in the NIP, some random hydrogen bonding might still occur.

4.6: Wash solvent optimization

The wash solvent optimization results, from Section 3.7, are presented and discussed in this section. The aim of these experiments was to establish an optimal wash solvent, comprised of a determined fraction of methanol in water for a controlled polarity. The wash solvent refers to the solution used to wash the sorbent after the loading fraction has passed through the SPE, prior to elution. The experiments are divided into subsections of the sorbent type used namely: C18, NIP and triazine and chloroacetamide MIP sorbents.

4.6.1: C18 SPE methanol: water wash solvent gradient

A solvent gradient of methanol: water was passed through the C18 SPE cartridge after it was loaded with 3 mL of 16.67 µg/mL for each pesticide (refer to Section 3.7.1). Table 4.49 displays all the 3 mL fractions and their respective pesticide concentrations measured over the solvent gradient collected from the C18 SPE.

Table 4.49: Results obtained for the C18 SPE methanol: water wash solvent optimization

Fraction #	Volume (mL)	Methanol (%)	Atrazine (µg/mL)	Terbuthylazine (µg/mL)	Acetochlor (µg/mL)	Alachlor (µg/mL)
1 (Load)	3	3.3	0	0	0	0
2	3	10	0	0	0	0
3	3	15	0	0	0	0
4	3	20	0	0	0	0
5	3	25	0	0	0	0
6	3	30	0.06	0	0	0
7	3	35	10.39	0	0	0
8	3	40	5.40	0.33	0	0
9	3	45	0.17	4.50	0	0
10	3	50	0	12.80	4.30	2.89
11	3	60	0	1.02	10.80	11.50
12	3	65	0	0	0.20	0.30
13	3	70	0	0	0	0
14	3	75	0	0	0	0
15	3	80	0	0	0	0

A visual comparison of the fractions in Table 4.49, is presented in Figure 4.44.

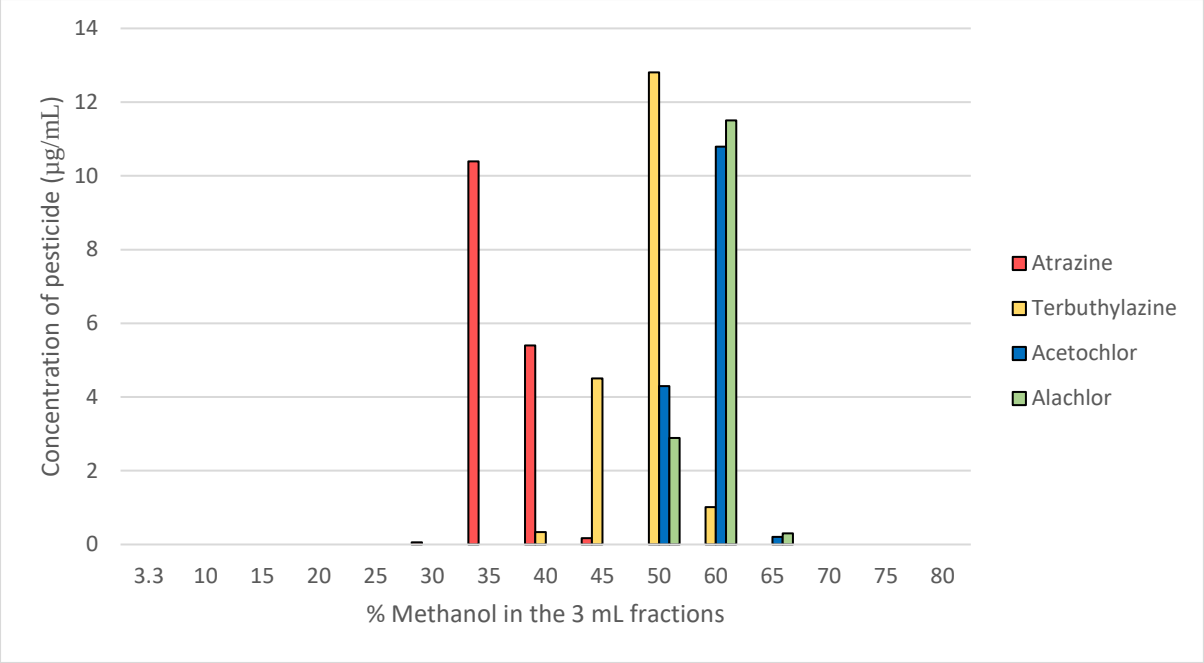


Figure 4.44: Pesticide concentrations in the different solvent fractions (3 mL) from the C18 SPE

From Figure 4.44 it seems very possible to separate atrazine and terbuthylazine from the chloroacetanilide pesticides by using a 45% methanol solution as wash solvent on the C18 sorbent. It was interesting to note the elution order does not correlate with log K_{ow} values, although atrazine which has the lowest log K_{ow} of 2.65 and alachlor the highest, at 3.52, do elute in the expected order. However, terbuthylazine and acetochlor are in a reverse order of what would be expected. Acetochlor elutes after terbuthylazine, despite having the lower log K_{ow} value of 3.03. This indicates that perhaps there are other factors to consider besides the octanol water partition coefficient for the retention of pesticides on the C18 sorbent. A 20 to 25% methanol solution would be an optimal solution to use for a wash solvent, as this was found to be the maximum concentration where none of the pesticides eluted yet. If only acetochlor and alachlor were targeted, a solution as high 45% methanol could be considered for the wash solvent of choice.

4.6.2: NISPE methanol: water wash solvent gradient

Table 4.50 displays all the fractions and their respective pesticide concentrations over the solvent gradient collected from the NISPE (refer to Section 3.7.2). It was found necessary to start at a much lower concentration of methanol for the NISPE than for the C18 SPE experiment in discussed Section 4.6.1. Subsequently a 10 mL loading fraction was prepared at 1% methanol (100 µL pesticide working standard and 9.9 mL water). All the subsequent wash fractions had a volume of 3 mL.

Table 4.50: Results obtained for the NISPE methanol: water wash solvent optimization experiment

Fraction #	Methanol (%)	Volume (mL)	Atrazine (µg/mL)	Terbuthylazine (µg/mL)	Acetochlor (µg/mL)	Alachlor (µg/mL)
1 (Load)	1	10	0.48	0.17	0.14	0.27
2	2	3	0.17	0.27	2.51	2.29
3	4	3	0.19	0.28	6.04	4.83
4	6	3	0.62	0.33	3.61	3.83
5	8	3	0.93	0.56	2.46	2.29
6	10	3	0.87	0.56	0.20	0.35
7	12	3	0.98	0.76	0.31	0.50
8	14	3	0.94	0.81	0.17	0.29
9	16	3	0.78	0.60	0.16	0.26
10	18	3	0.72	0.90	0.32	0.50
11	20	3	0.66	1.57	0.24	0.35
12	30	3	1.21	2.86	0.31	0.40
13	40	3	1.73	4.74	0.10	0.20
14	50	3	5.88	2.32	0.13	0.17
15	60	3	1.65	0.90	0.09	0.00
16	70	3	0.03	0.28	0.05	0.00
17	80	3	0	0.15	0	0

A visual comparison of each fraction was presented in Figure 4.45 for each measured pesticide concentration in every methanol fraction.

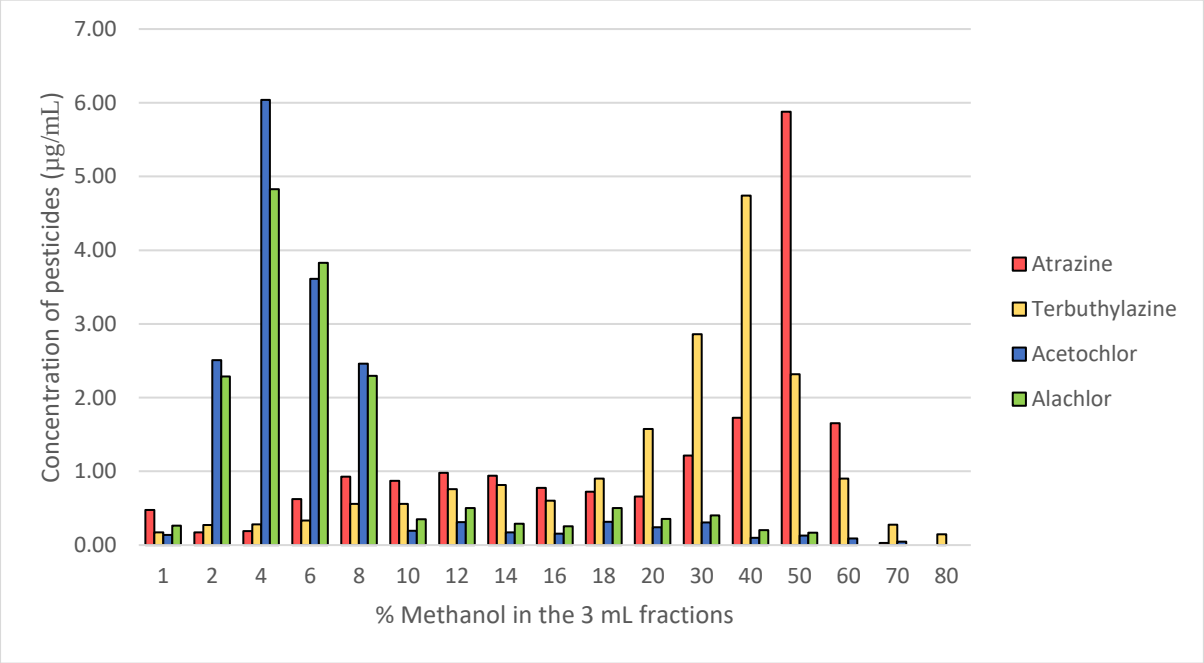


Figure 4.45: Pesticide concentrations obtained in the different solvent fractions (3 mL) from the NISPE

A bleeding effect was observed from the loading fraction at 1% methanol, where low concentrations of all four pesticides were observed to have passed through the NISPE cartridge. This was most likely because the NISPE was not packed as tightly as the commercial C18 SPE sorbent, thus some small channels were present allowing a small fraction of the pesticide to pass through uninhibited. Acetochlor and alachlor clearly eluted from the cartridge first, with the bulk of these two pesticides eluting between 2 and 8% methanol. Considering the higher water solubility of acetochlor and alachlor this was not found to be surprising, although they have higher log K_{OW} values than atrazine (Section 2.2.4). Terbuthylazine has the highest concentration at 40% methanol and atrazine at 50% methanol wash solution. It was observed that the elution order was almost the exact reverse from the C18 SPE experiment discussed in Section 4.6.1, where atrazine eluted first and acetochlor and alachlor last. This indicates a more complex adsorption mechanism than just hydrophobicity. Acetochlor and alachlor have fewer places for hydrogen bonding, as depicted in Figure 2.46 and Figure 2.47 when possible orientations of MAA and EDGMA were considered for MIP synthesis (Section 2.7.2.3), whereas atrazine and terbuthylazine have more functional groups where hydrogen bonding with MAA can occur thus providing a possible explanation of their stronger retention on the NIP.

4.6.3: Triazine MISPE methanol: water wash solvent gradient

Table 4.51 displays all the fractions and their respective concentrations over the solvent gradient collected from both triazine MISPEs, where atrazine and terbuthylazine are displayed in separate columns (refer to Section 3.7.3). All the fractions, including the loading fraction, were made up to a volume of 3 mL.

Table 4.51: Results obtained for the triazine MISPE methanol: water wash solvent optimization experiment

Fraction #	Methanol (%)	Volume (mL)	Atrazine on atrazine MISPE (µg/mL)	Terbuthylazine on terbuthylazine MISPE (µg/mL)
1 (Load)	0.83	3	0	0
2	10	3	0	0
3	12	3	0.04	0.03
4	14	3	0.10	0.00
5	16	3	0.38	0.04
6	18	3	0.84	0.35
7	20	3	0.85	0.49
8	22	3	0.90	1.11
9	24	3	1.11	1.18
10	26	3	1.15	1.58
11	28	3	1.34	2.37
12	30	3	2.30	2.77
13	40	3	2.89	4.34

Fraction #	Methanol (%)	Volume (mL)	Atrazine on atrazine MISPE (µg/mL)	Terbuthylazine on terbuthylazine MISPE (µg/mL)
14	50	3	3.59	1.78
15	60	3	0.61	0.00
16	70	3	0	0.00
17	80	3	0	0.00

Bar graphs for atrazine (Figure 4.46) and terbuthylazine (Figure 4.47) respectively, represent the data from Table 4.51 in a visual manner.

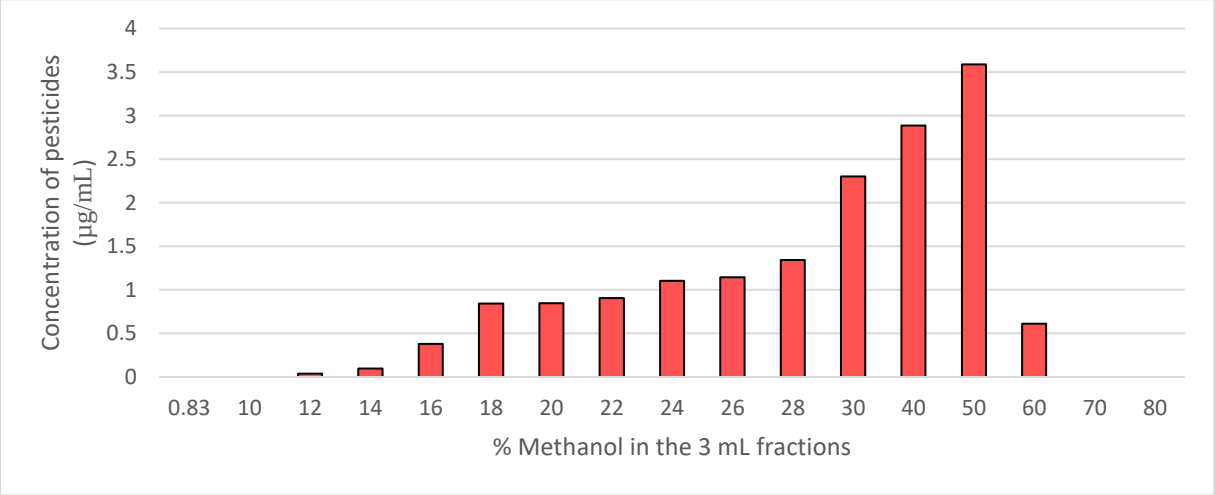


Figure 4.46: Atrazine concentrations obtained in 3 mL solvent fractions from a packed atrazine MISPE

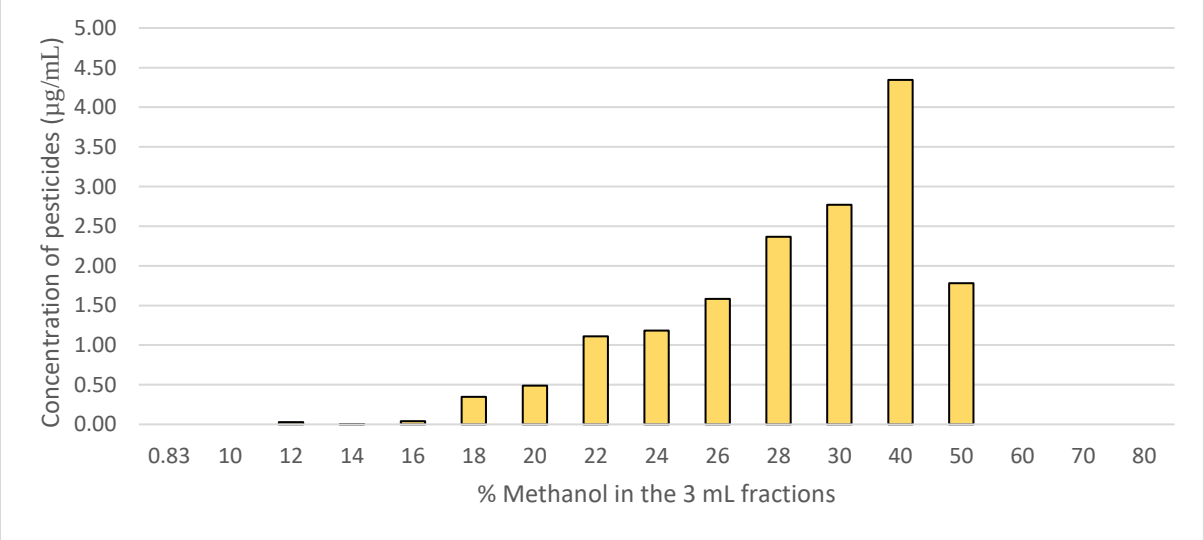


Figure 4.47: Terbuthylazine concentrations obtained for each of the 3 mL solvent fractions from a terbuthylazine MISPE

There was no atrazine or terbuthylazine detected in the first and last two methanol: water fractions as observed in Table 4.51. A significant concentration of atrazine was first observed in the 16% methanol fraction and at 18% methanol for terbuthylazine. Unlike the NISPE, no pesticide was observed in the loading fraction indicating a stronger retention or less channelling. The pesticide

concentration increased gradually with the increase in methanol content, until presumably there was no more pesticide left on the sorbent that could be eluted.

4.6.4: Chloroacetamide MISPE methanol: water wash solvent gradient

Table 4.52 displays all the fractions and their respective concentrations of pesticides over the methanol solvent gradient collected from both chloroacetamide MISPEs and their respective template pesticides. The methodology was discussed in Section 3.7.4. All the fractions, including the loading fraction, was made up to a 3 mL volume with the concentration for both the acetochlor and alachlor loading fractions at 16.67 µg/mL.

Table 4.52: Results obtained for the chloroacetamide MISPE methanol: water wash solvent optimization experiment

Fraction #	Methanol (%)	Volume (mL)	Acetochlor on acetochlor MISPE (µg/mL)	Alachlor on alachlor MISPE (µg/mL)
1 (Load)	0,83	3	0	0
2	2	3	0	0
3	4	3	0	0,5
4	6	3	0,5	2,9
5	8	3	4,3	3,5
6	10	3	5,4	3,8
7	12	3	2,5	3,2
8	14	3	1,8	1,8
9	16	3	1,1	0,9
10	18	3	0,4	0,4
11	20	3	0,1	0,2
12	30	3	0	0
13	40	3	0	0
14	50	3	0	0
15	60	3	0	0
16	70	3	0	0
17	80	3	0	0

The two bar graphs displayed in Figure 4.48 and Figure 4.49 visually represent the data for each fraction in Table 4.52 for acetochlor and alachlor with their complimentary MISPEs.

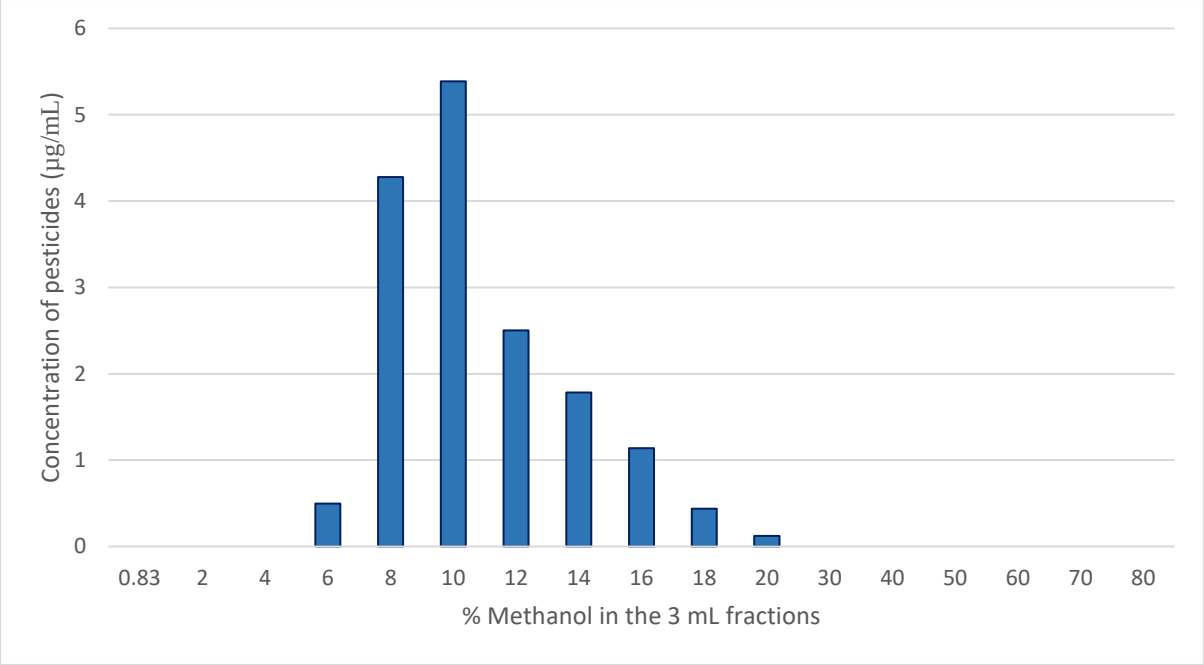


Figure 4.48: Acetochlor concentrations obtained for each of the 3 mL solvent fractions from an acetochlor MISPE

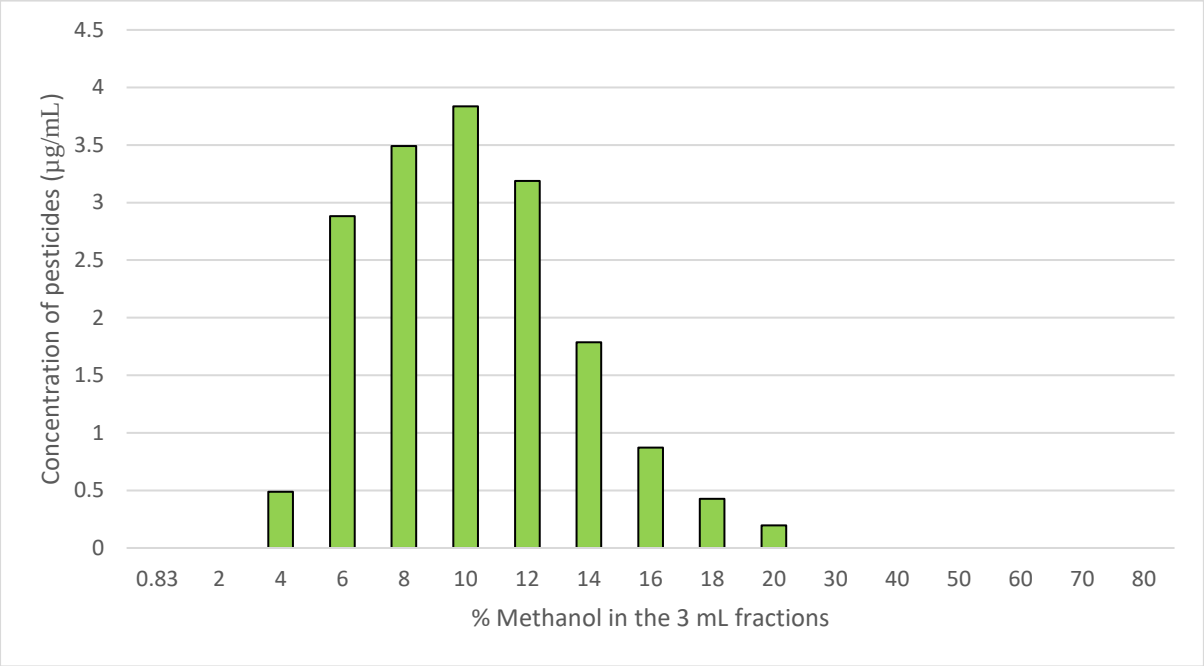


Figure 4.49: Alachlor concentrations obtained for each of the 3 mL solvent fractions from an alachlor MISPE

There was no acetochlor or alachlor detected in the first methanol: water fractions as observed in Table 4.52. A significant concentration of alachlor was first observed in the 4% methanol wash fraction and at 6% methanol for acetochlor on each of their respective MISPEs. Unlike the NISPE, no pesticide

was observed in the loading fraction indicating a stronger retention in the orientation specific cavities or less channelling in the MISPE packing. The pesticide concentration increased gradually with the increase in methanol, until presumably there was no more pesticide left on the sorbent that could be eluted, then the concentration decreased slowly.

4.6.5: MTMIP wash solvent optimization

Table 4.53 and Figure 4.50 display all the fractions and the respective pesticide concentrations over the solvent gradient collected from the MTMISPE. The methodology was discussed in Section 3.7.5. All the fractions, except for the loading fraction, were made up to a 3 mL volume with the concentration for each pesticide in loading fraction at 16.67 µg/mL.

Table 4.53: Results obtained for all the pesticides on the MTMISPE for the methanol: water wash solvent optimization experiment

Fraction #	Methanol (%)	Volume (mL)	Atrazine on MTMISPE (µg/mL)	Terbuthylazine on MTMISPE (µg/mL)	Acetochlor on MTMISPE (µg/mL)	Alachlor on MTMISPE (µg/mL)
1 (Load)	1	10	0	0	0	0
2	2	3	0	0	0	0
3	4	3	0	0	0	0.43
4	6	3	0	0	0.46	2.56
5	8	3	0	0	3.29	3.10
6	10	3	0	0	3.99	3.41
7	12	3	0.04	0.03	2.63	2.83
8	14	3	0.10	0.00	1.97	1.59
9	16	3	0.38	0.04	1.37	0.77
10	18	3	0.84	0.35	0.40	0.38
11	20	3	0.85	0.49	0.14	0.17
12	22	3	0.90	1.11	0.13	0.09
13	24	3	1.11	1.18	0	0
14	26	3	1.15	1.58	0	0
15	28	3	1.34	2.37	0	0
16	30	3	2.30	2.77	0	0
17	40	3	2.89	3.69	0	0
18	50	3	3.59	1.78	0	0
19	60	3	0.61	0	0	0
20	70	3	0	0	0	0
21	80	3	0	0	0	0

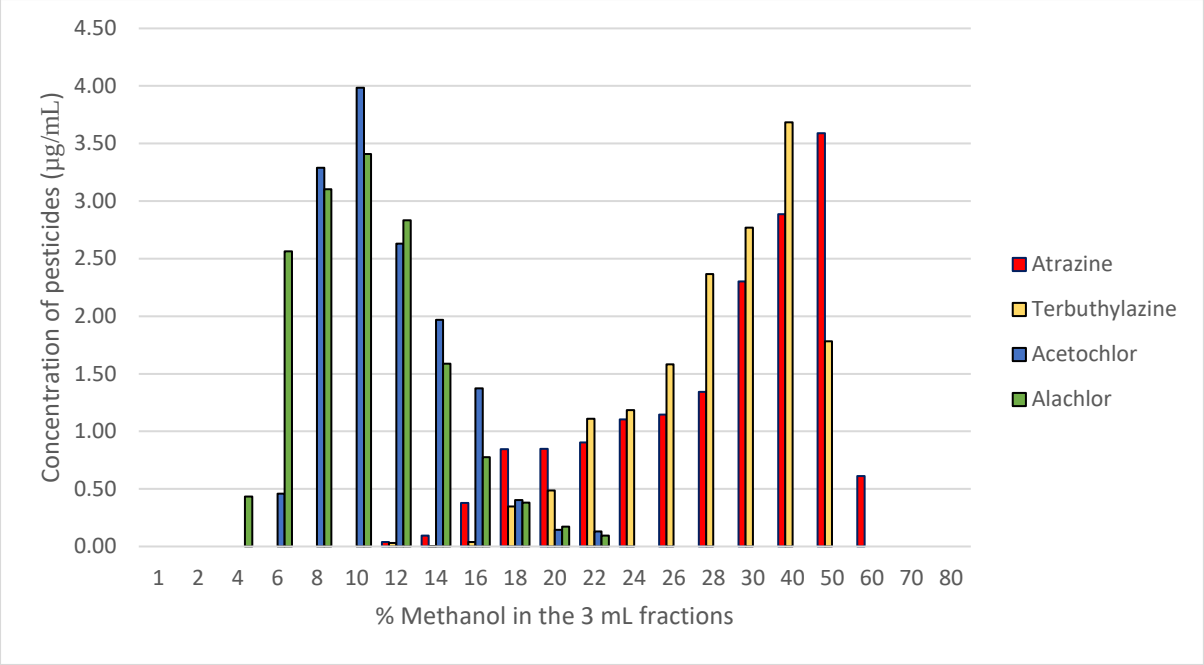


Figure 4.50: Pesticide concentrations obtained for each of the solvent fractions from a MTMISPE cartridge

From the elution data obtained, displayed in Figure 4.50 and Table 4.53, there appears to be very little difference between the MTMIP and the previous triazine and chloroacetamide MIPs reported in Section 4.6.3 and 4.6.4 respectively. Significant concentrations of acetochlor and alachlor were detected in the 6 and 4% methanol fractions, respectively. A concentration of 2% methanol can thus be used as a wash solvent, as no alachlor was detected in this fraction.

4.7: Recovery determination on spiked cannabis flower samples

This section presents results obtained for spiked cannabis flower samples extracted with two different techniques and subsequently pre-concentrated and cleaned up employing several variations of SPE, including C18, NIP and MIP sorbents. The methodology was discussed in Section 3.8 for all the extraction and SPE procedures.

Figure 4.51 was taken after approximately 2 g of plant material underwent the salting out procedure in a 15 mL PTFE tube. Less sample was used for this purpose so that the layers could be clearly seen. Acetonitrile has a lower density than water and was thus the top fraction. Plant material can be observed in two major layers, below and above the water fraction. There is also a seed floating in the top right section of the acetonitrile fraction in Figure 4.51. The undissolved NaCl salt is at the very bottom of the SPE tube.

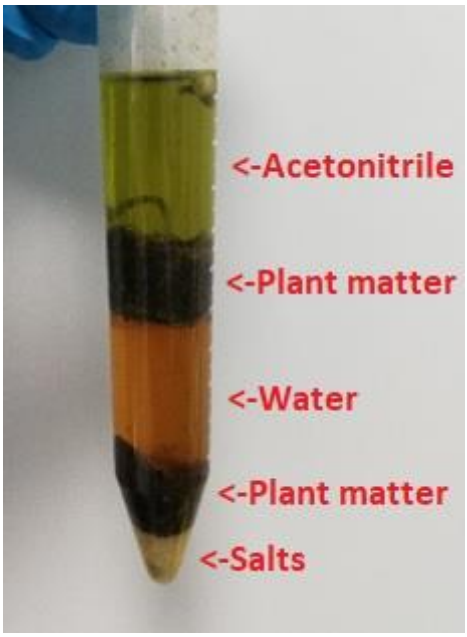


Figure 4.51: Layers observed once acetonitrile and salt extraction was performed on the cannabis plant material

Approximately 3 g of fine plant material was weighed out into a 50 mL PTFE centrifuge tube and spiked with pesticides to reach the desired MRL and 20 mL water was add/ed. Figure 4.52 shows an example sample after centrifugation.

Figure 4.52: The 50 mL PTFE centrifuge tube with water and approximately 3 g cannabis buds after centrifugation

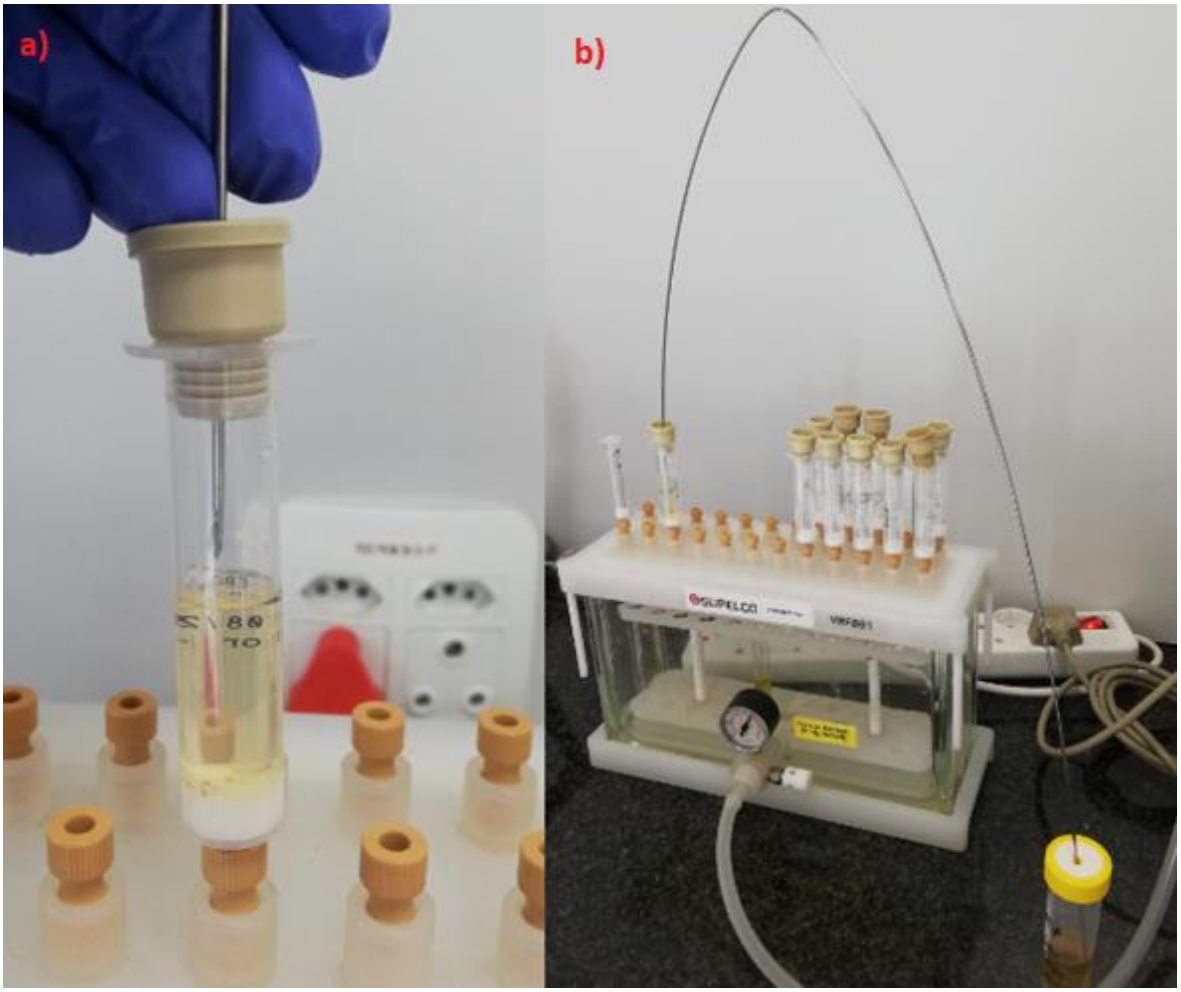


Figure 4.53: (a) A double sided needle and rubber septum being used to pass a large aqueous sample through an SPE cartridge and (b) shows the entire needle, from sample to SPE cartridge

The water loading fraction was observed to pass through the sorbents (NIP, all MIPs and C18) far easier than the acetonitrile extracts. Although the loading fraction was much larger for the water extractions, they could pass through the SPEs much faster and with less vacuum.

4.7.1: Matrix matched calibration curves at lower concentrations

The following calibration curves were obtained for each pesticide used as a template during MIP synthesis utilizing matrix matched reconstitution of the calibration standards. The methodology for the matrix matched calibration was discussed in Section 3.8.1. Figures 4.51 to 4.54 contain calibration curves for atrazine, terbuthylazine, acetochlor and alachlor respectively, including the best fit lines and correlation coefficients (R^2). Area ratio was plotted on the Y axis and the concentration in µg/mL (ppm) on the X axis. Area ratio was employed due the internal standard (IS) employed and represents the peak area of the analyte divided by the IS peak area.

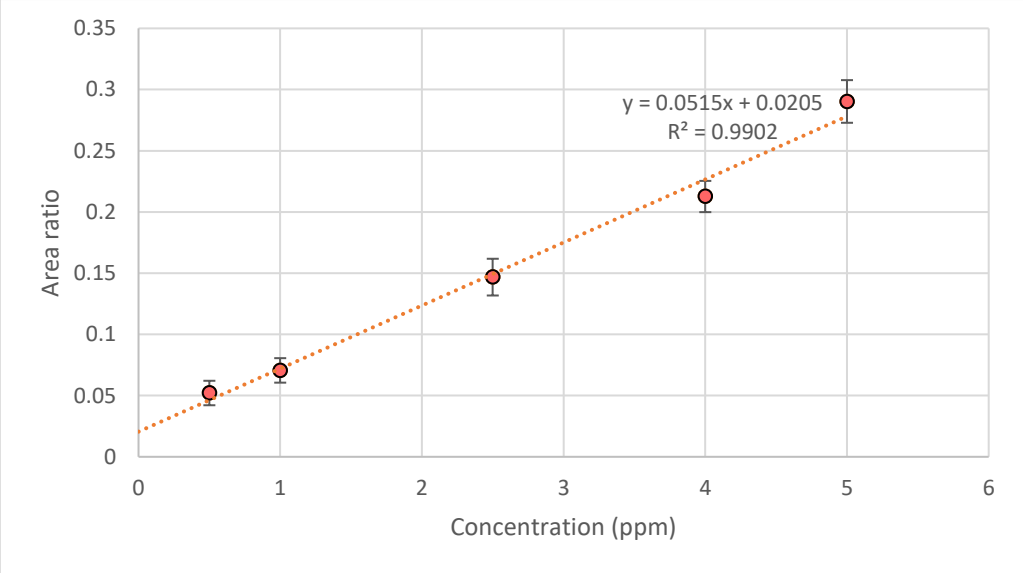


Figure 4.54: Atrazine matrix matched calibration curve with the lowest concentration at 0.5 µg/mL. The error bars represent the calculated standard deviation (N=3)

The R^2 value of the calibration curve for atrazine (Figure 4.54) was found to be excellent, being > 0.99, and the x intercept was found to be relatively close to zero, at -0.40 ppm.

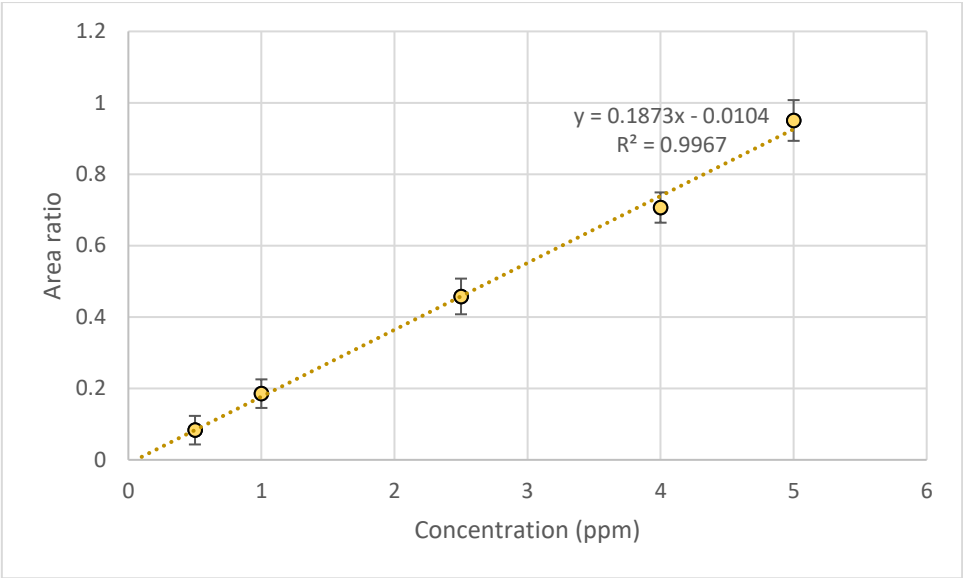


Figure 4.55: Terbuthylazine matrix matched calibration curve with the lowest concentration at 0.5 µg/mL. The error bars represent the calculated standard deviation (N=3)

The R^2 value was greater than 0.99, indicating a strong linear fit, for the terbuthylazine calibration curve. The line of best fit was not forced through zero (Figure 4.55). Drying and reconstitution with a matrix matched solution can introduce more variance to the method, resulting in the slightly larger error bars, especially at the lowest two concentrations of 0.5 and 1 ppm.

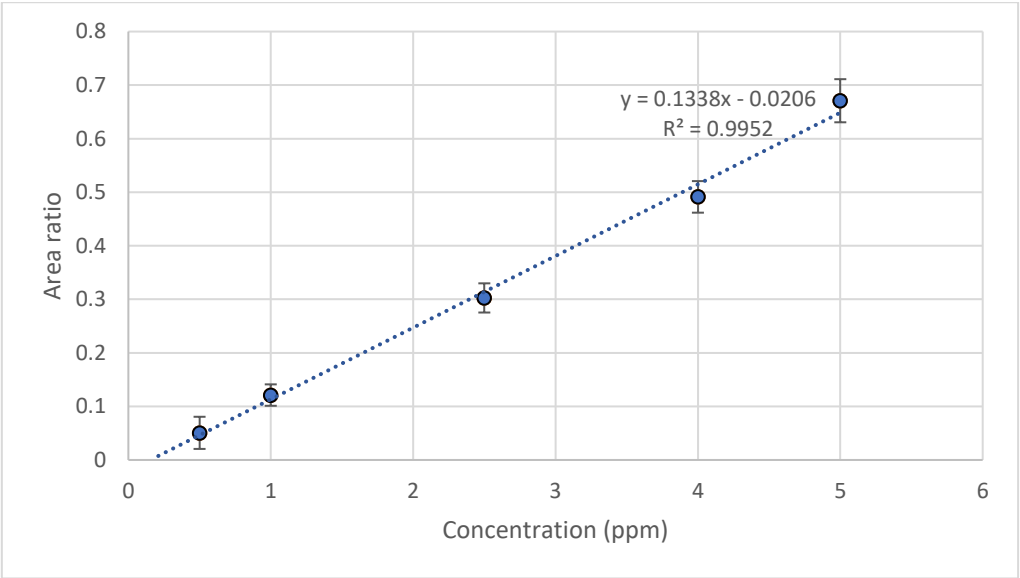


Figure 4.56: Acetochlor matrix matched calibration curve with the lowest concentration at 0.5 µg/mL. The error bars represent the calculated standard deviation (N=3)

The R^2 value was found to be greater than 0.99 indicating a strong linear fit for the acetochlor curve. The line of best fit was not forced through zero (Figure 4.56). The matrix matched reconstitution thus has a significant effect on the x intercept, at 0.15 ppm, that can be seen as a positive bias.

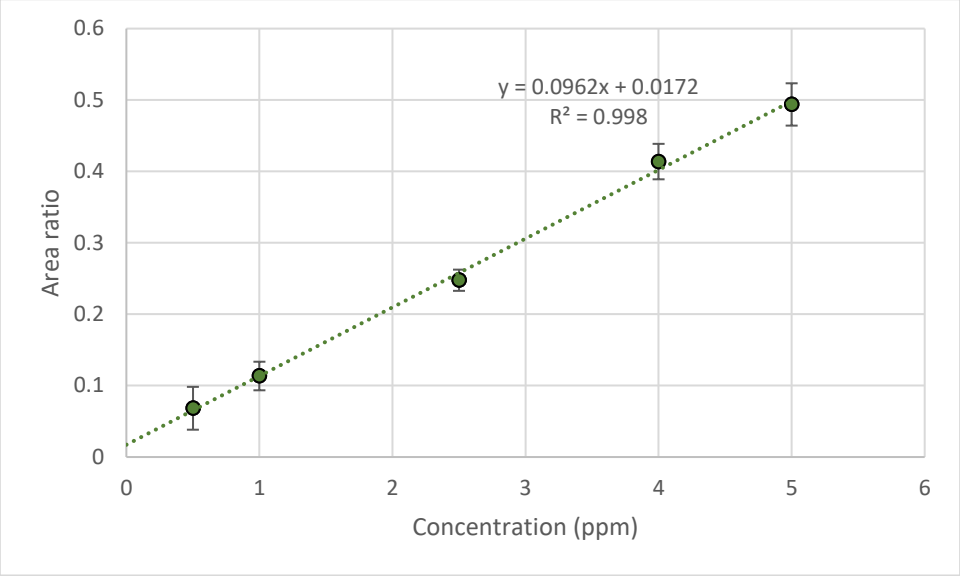


Figure 4.57: Alachlor matrix matched calibration curve with the lowest concentration at 0.5 µg/mL. The error bars represent the calculated standard deviation at each concentration (N=3)

The correlation coefficient (R^2) for all the calibration curves were close to one, including the alachlor matrix matched calibration curve represented in Figure 4.57.

The x intercept for all the curves are shown in Table 4.54. It can be seen that the sample matrix did not significantly affect all of the analytes in terms of their x intercept, although atrazine was the most affected by the matrix. In effect, the matrix matched calibration can be seen as a standard addition (although it will calculate a negative value in some cases) and the interferences of the sample matrix amount to a quantifiable bias result on the GC-MS method discussed in Section 3.2 proving the need for sample clean-up for extracts from the cannabis sample matrix especially when analysing lower concentrations.

Table 4.54: X intercepts for the matrix matched calibration curves for each pesticide

Analyte	Calibration line	x intercept (y=0) ppm
Atrazine	y= 0.0515x+0.0205	-0.40
Terbuthylazine	y= 0.1873x-0.0104	0.06
Acetochlor	y= 0.1338x-0.0206	0.15
Alachlor	y= 0.0962x+0.0172	-0.18

4.7.1.1: Selectivity of GC-MS for the pesticide analytes in the cannabis flower sample matrix

The following chromatograms (Figure 4.58 to 4.62) were taken from the unspiked methanol cannabis extraction used to reconstitute the matrix matched calibration standards after the drying step. The retention window was indicated on each quantitation SIR chromatogram, and only atrazine had a small integrated interference with the integration parameters used for the calibration curves. These chromatograms served to prove the selectivity of the GC-MS instrumental method for the selected pesticides as no additional major peaks from the cannabis matrix were observed in the indicated retention time windows.

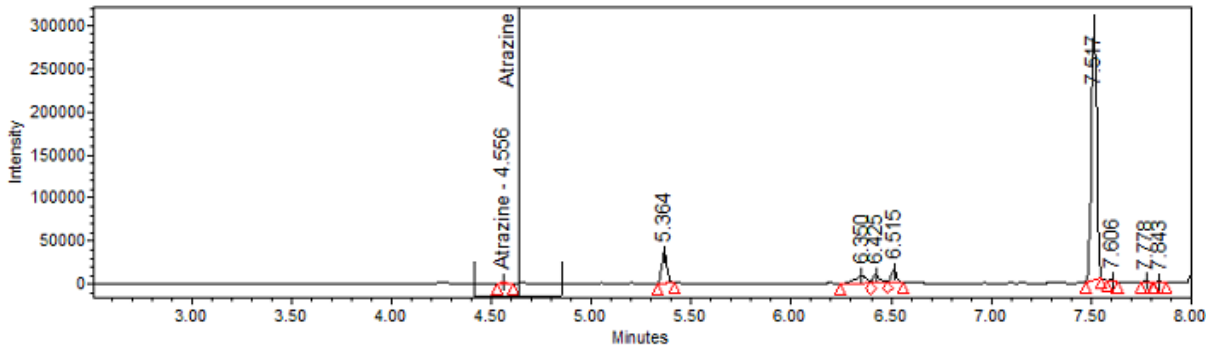


Figure 4.58: SIR chromatogram showing the atrazine (m/z 200 Da) retention time window for the un-spiked methanol cannabis flower extraction

The integrated peak (Figure 4.58) was close to the atrazine peak, and falls in the retention time window set up for atrazine quantitation, but does not contain the corresponding qualification ion (Figure 4.59) on the *m/z* 215 chromatogram, proving the integrated peak cannot be identified as atrazine.

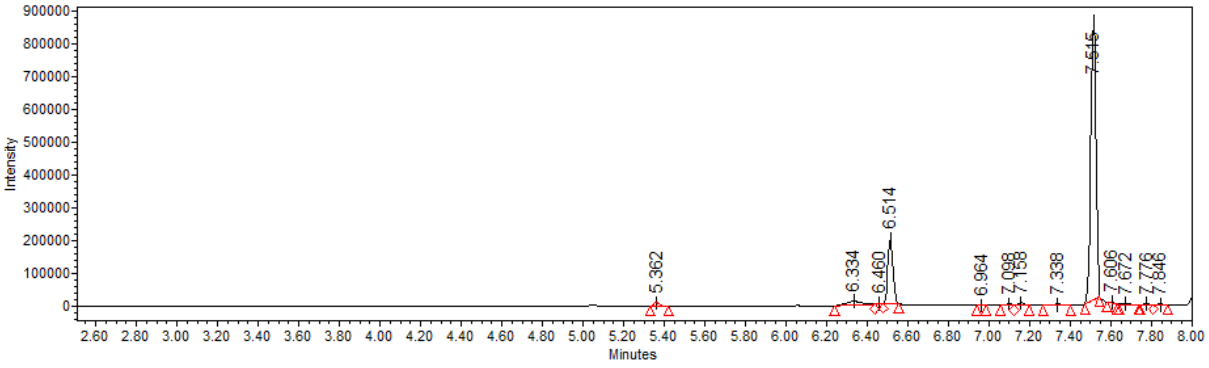


Figure 4.59: Atrazine qualification SIR chromatogram (m/z 215 Da) on the un-spiked methanol cannabis flower extract, that shows no peak at 4.55 min

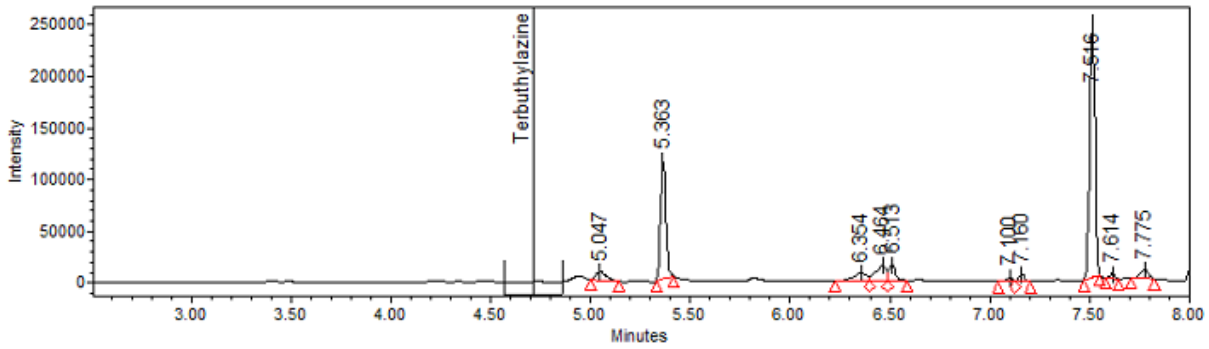


Figure 4.60: SIR chromatogram showing the terbuthylazine (m/z 214 Da) retention time window on the unspiked methanol cannabis flower extract

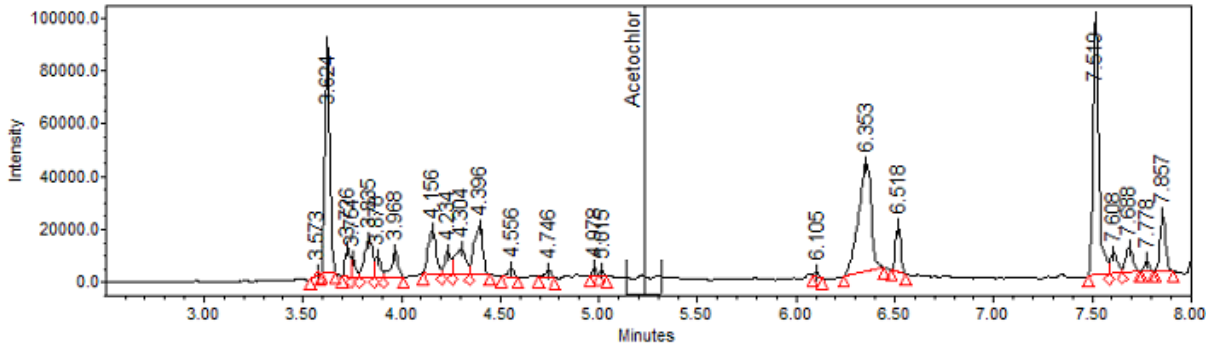


Figure 4.61: SIR chromatogram showing acetochlor (m/z 146 Da) retention time window on the unspiked methanol cannabis flower extract

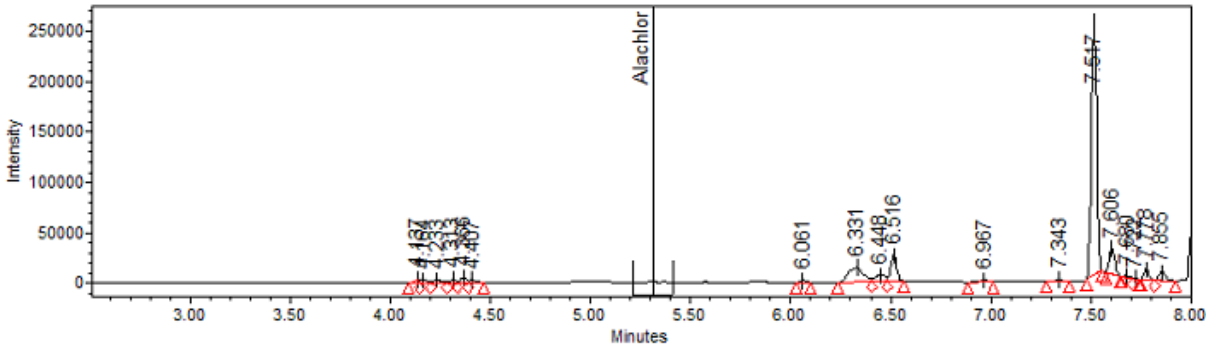


Figure 4.62: SIR chromatogram showing alachlor (m/z 160 Da) retention time window on the unspiked methanol cannabis flower extract

4.7.2: Recovery of spiked cannabis flowers on C18 SPE

Table 4.55 contains the results for the C18 SPE experiments performed as discussed in Section 3.8. The experimental summary was provided in Table 3.20, including the mass of each cannabis flower sample, the amount of spiked WS added and the loading, wash and elution fractions used during the SPE method. Table 4.55 contains three replicates for each pesticide for each extraction method. The theoretical concentration of each pesticide after reconstitution was calculated for each separate sample based on the sample mass, as shown in Table 4.55. All the samples were spiked at 0.05 µg/g as per the South African MRL for the selected pesticides on general crops, such as maize. The % standard deviation (STDEV) mean of the measured triplicate results and %RSD were calculated for each triplicate result in terms of % recovery. It was found necessary to reconstitute the acetonitrile extractions with 150 µL methanol, as the residue after evaporation was too much to fully dissolve in the planned 50 µL reconstitution solution. The water extractions were found to be cleaner.

Table 4.55: C18 SPE results obtained from spiked cannabis samples

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration on GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
1	Atrazine	Acetonitrile & NaCl	1.05	0.73	**69.9**	**9.7**	**83.5**	**11.6**
2	Atrazine	Acetonitrile & NaCl	1.08	0.97	**89.1**			
3	Atrazine	Acetonitrile & NaCl	1.02	0.95	**91.5**			
1	Terbuthylazine	Acetonitrile & NaCl	1.05	0.93	**92.6**	**9.7**	**80.7**	**12.1**
2	Terbuthylazine	Acetonitrile & NaCl	1.08	0.70	**68.8**			
3	Terbuthylazine	Acetonitrile & NaCl	1.02	0.83	**80.8**			
4	Acetochlor	Acetonitrile & NaCl	1.01	1.10	**109.2**	**10.6**	**96.2**	**11.0**
5	Acetochlor	Acetonitrile & NaCl	1.00	0.86	**83.3**			
6	Acetochlor	Acetonitrile & NaCl	1.01	0.91	**96.1**			
4	Alachlor	Acetonitrile & NaCl	1.01	1.22	**115.1**	**13.7**	**96.9**	**14.1**
5	Alachlor	Acetonitrile & NaCl	1.00	0.94	**93.6**			
6	Alachlor	Acetonitrile & NaCl	1.01	0.87	**82.0**			

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration on GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
7	Atrazine	Water rinsing	3.08	2.02	**64.9**	**8.3**	**76.6**	**10.8**
8	Atrazine	Water rinsing	3.19	2.63	**81.5**			
9	Atrazine	Water rinsing	3.24	2.76	**83.3**			
7	Terbuthylazine	Water rinsing	3.08	2.58	**78.0**	**9.6**	**77.1**	**12.5**
8	Terbuthylazine	Water rinsing	3.19	2.11	**64.9**			
9	Terbuthylazine	Water rinsing	3.24	2.95	**88.4**			
10	Acetochlor	Water rinsing	2.96	3.03	**101.3**	**6.9**	**91.6**	**7.6**
11	Acetochlor	Water rinsing	2.94	2.62	**88.4**			
12	Acetochlor	Water rinsing	3.17	2.77	**85.2**			
10	Alachlor	Water rinsing	2.96	3.41	**103.5**	**6.7**	**95.3**	**7.1**
11	Alachlor	Water rinsing	2.94	3.12	**95.3**			
12	Alachlor	Water rinsing	3.17	2.67	**87.0**			

The standard deviation and mean of the triplicate results are displayed in Table 4.55, and t-tests were performed to compare the different extraction methods. The t_{crit} value was found to be 2.776 at 95% confidence and degrees of freedom of 4 (n_1+n_2-2). The t-test results are presented in Table 4.56.

Table 4.56: T-tests for the C18 SPE pesticide recoveries from cannabis flower extracts

Group A	Group B	Calculated t value
Atrazine (Acetonitrile & NaCl)	Terbuthylazine (Acetonitrile & NaCl)	0.412
Atrazine (Acetonitrile & NaCl)	Acetochlor (Acetonitrile & NaCl)	1.767
Atrazine (Acetonitrile & NaCl)	Alachlor (Acetonitrile & NaCl)	1.595
Terbuthylazine (Acetonitrile & NaCl)	Acetochlor (Acetonitrile & NaCl)	2.154
Terbuthylazine (Acetonitrile & NaCl)	Alachlor (Acetonitrile & NaCl)	1.926
Acetochlor (Acetonitrile & NaCl)	Alachlor (Acetonitrile & NaCl)	0.083
Atrazine (Water rinsing)	Terbuthylazine (Water rinsing)	0.083
Atrazine (Water rinsing)	Acetochlor (Water rinsing)	**2.784**
Atrazine (Water rinsing)	Alachlor (Water rinsing)	**3.507**
Terbuthylazine (Water rinsing)	Acetochlor (Water rinsing)	2.449
Terbuthylazine (Water rinsing)	Alachlor (Water rinsing)	**3.100**
Acetochlor (Water rinsing)	Alachlor (Water rinsing)	0.757

Group A	Group B	Calculated t value
Atrazine (Acetonitrile & NaCl)	Atrazine (Water rinsing)	1.095
Terbuthylazine (Acetonitrile & NaCl)	Terbuthylazine (Water rinsing)	0.530
Acetochlor (Acetonitrile & NaCl)	Acetochlor (Water rinsing)	0.722
Alachlor (Acetonitrile & NaCl)	Alachlor (Water rinsing)	0.212

In Table 4.56, values where the calculated t value was found to be greater than the t_{crit} value are in bold.

If a 95% confidence interval was used (t_{crit} = 2.776) none of the compared groups tested for the acetonitrile & NaCl extractions were found to be statistically different. There also appeared to be no statistical difference between the water rinsing and salted acetonitrile extraction methods for the individual pesticides. Only when comparing atrazine and terbuthylazine with acetochlor and alachlor, both extracted with repeated water rinsing, were there some statistical differences observed. From the means in Table 4.55, acetochlor and alachlor extracted from the cannabis samples constantly have higher recoveries on the C18 SPE than the two triazine pesticides. This statistical difference can be explained by the higher affinity of the C18 sorbent for acetochlor and alachlor during the wash solvent optimization experiments in Section 4.6.1. Another factor that had to be considered was that acetochlor and alachlor have a higher water solubility as discussed in Section 2.2.4, which was most probably the reason for the higher observed recovery of the chloroacetamide pesticides when using water rinsing extraction, along with a greater affinity for the C18 sorbent when compared to the triazine pesticides.

4.7.3: Recovery of spiked cannabis flowers on NISPE

As explained in Section 3.8.4, at the lower concentrations of 0.05 mg/kg, some of the triplicate NISPE experiments had no detected chloroacetamide pesticides. The data obtained for all the NISPEs from spiked cannabis sample matrix are displayed in Table 4.57 and correlate to the procedure summarised in Table 3.20. Replicates 1 to 12 were spiked at 0.05 mg/kg and 13 to 22 were spiked at 0.5 mg/kg. A lot less sample was weighed, and half the solvent volumes were used as compared to the replicates spiked at 0.5 mg/kg.

It was not found necessary to reconstitute the acetonitrile extractions with 150 µL methanol as with the C18 sorbent, however to be able to compare the synthesised sorbents with the C18 SPE the reconstitution volume was kept constant, and the theoretical 100% extraction concentration after reconstitution was thus approximately 1 µg/mL, as indicated in Table 4.57.

Table 4.57: Result summary for NISPE extraction performed from cannabis plant material

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
1	Atrazine	Acetonitrile & NaCl	1.01	0.19	18.9	3.6	18.6	19.2
2	Atrazine	Acetonitrile & NaCl	1.00	0.14	14.1			
3	Atrazine	Acetonitrile & NaCl	1.01	0.23	22.8			
1	Terbuthylazine	Acetonitrile & NaCl	0.99	0.17	17.2	1.9	18.9	10.1
2	Terbuthylazine	Acetonitrile & NaCl	1.00	0.18	18.0			
3	Terbuthylazine	Acetonitrile & NaCl	1.02	0.22	21.6			
4	Acetochlor	Acetonitrile & NaCl	1.01	0	0.0	N/A	0.0	N/A
5	Acetochlor	Acetonitrile & NaCl	1.06	0	0.0			
6	Acetochlor	Acetonitrile & NaCl	1.01	0	0.0			
4	Alachlor	Acetonitrile & NaCl	0.98	0	0.0	N/A	0.0	N/A
5	Alachlor	Acetonitrile & NaCl	1.01	0	0.0			
6	Alachlor	Acetonitrile & NaCl	1.00	0	0.0			
7	Atrazine	Water rinsing	3.08	0.63	20.5	2.1	23.1	9.0
8	Atrazine	Water rinsing	2.93	0.75	25.6			
9	Atrazine	Water rinsing	3.17	0.74	23.3			
7	Terbuthylazine	Water rinsing	2.92	0.91	31.2	5.2	23.8	22.1
8	Terbuthylazine	Water rinsing	2.91	0.58	19.9			
9	Terbuthylazine	Water rinsing	3.06	0.62	20.2			
10	Acetochlor	Water rinsing	2.98	0.00	0.0	N/A	0.0	N/A
11	Acetochlor	Water rinsing	3.04	0.00	0.0			
12	Acetochlor	Water rinsing	2.91	0.00	0.0			
10	Alachlor	Water rinsing	3.16	0.00	0.0	N/A	0.0	N/A
11	Alachlor	Water rinsing	3.01	0.00	0.0			
12	Alachlor	Water rinsing	3.17	0.00	0.0			
13	Atrazine	Acetonitrile & NaCl	0.99	0.63	63.5	7.6	70.3	10.8
14	Atrazine	Acetonitrile & NaCl	1.04	0.84	80.8			
15	Atrazine	Acetonitrile & NaCl	1.05	0.70	66.6			
13	Terbuthylazine	Acetonitrile & NaCl	1.06	0.64	60.2	13.7	79.2	17.3
14	Terbuthylazine	Acetonitrile & NaCl	1.02	0.87	85.0			
15	Terbuthylazine	Acetonitrile & NaCl	1.04	0.96	92.2			
16	Acetochlor	Acetonitrile & NaCl	1.04	0.07	6.8	1.8	7.0	25.4
17	Acetochlor	Acetonitrile & NaCl	1.02	0.05	4.9			
18	Acetochlor	Acetonitrile & NaCl	0.98	0.09	9.2			
16	Alachlor	Acetonitrile & NaCl	1.03	0.04	3.9	1.3	5.6	23.0
17	Alachlor	Acetonitrile & NaCl	1.00	0.07	7.0			
18	Alachlor	Acetonitrile & NaCl	1.03	0.06	5.8			
19	Atrazine	Water rinsing	2.92	1.52	52.1	10.3	54.0	19.0
20	Atrazine	Water rinsing	3.06	2.06	67.4			
21	Atrazine	Water rinsing	3.34	1.42	42.5			
19	Terbuthylazine	Water rinsing	3.38	1.51	44.7	8.6	53.4	16.2
20	Terbuthylazine	Water rinsing	3.25	2.12	65.2			
21	Terbuthylazine	Water rinsing	3.31	1.67	50.4			
22	Acetochlor	Water rinsing	3.04	0.33	10.8	2.9	10.4	27.6
23	Acetochlor	Water rinsing	3.00	0.20	6.7			
24	Acetochlor	Water rinsing	3.07	0.42	13.7			

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
22	Alachlor	Water rinsing	3.23	0.24	7.4	1.7	7.9	22.0
23	Alachlor	Water rinsing	3.14	0.19	6.0			
24	Alachlor	Water rinsing	3.03	0.31	10.2			

The NISPE experiments were found to be far more difficult than that for the C18 SPE previously described. It was found necessary to spike at a higher concentration in some instances to be able to detect some pesticides post NISPE and reconstitution. The triplicate experiments were done simultaneously, thus as displayed in the results section some triplicate results have no detected pesticides.

From the data in Table 4.57 the standard deviation and average recovery values were calculated and t-tests were performed to compare the different triplicate results. The t_{crit} value was identified as 2.776 at a 95% confidence interval and 4 degrees of freedom. The t-test results are presented in Table 4.58. Only results of spiked samples at the same concentrations were compared. The acetochlor and alachlor that were not detected at the lower spiked concentration sample were not included in the t tests.

Table 4.58: T-tests for the pesticide recoveries from cannabis flower extracts cleaned utilising pre-treated NISPE

Group A	Group B	Calculated t-value
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg	Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg	1.113
Atrazine (Water rinsing) 0.05mg/kg	Terbuthylazine (Water rinsing) 0.05 mg/kg	0.233
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg	Atrazine (Water rinsing) 0.05mg/kg	2.211
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg	Terbuthylazine (Water rinsing) 0.05 mg/kg	1.744
Atrazine (Acetonitrile & NaCl) 0.5 mg/kg	Terbuthylazine (Acetonitrile & NaCl) 0.5 mg/kg	1.132
Atrazine (Acetonitrile & NaCl) 0.5 mg/kg	Acetochlor (Acetonitrile & NaCl) 0.5 mg/kg	**16.894**
Atrazine (Acetonitrile & NaCl) 0.5 mg/kg	Alachlor (Acetonitrile & NaCl) 0.5 mg/kg	**16.424**
Terbuthylazine (Acetonitrile & NaCl) 0.5 mg/kg	Acetochlor (Acetonitrile & NaCl) 0.5 mg/kg	**10.674**
Terbuthylazine (Acetonitrile & NaCl) 0.5 mg/kg	Alachlor (Acetonitrile & NaCl) 0.5 mg/kg	**10.463**
Acetochlor (Acetonitrile & NaCl) 0.5 mg/kg	Alachlor (Acetonitrile & NaCl) 0.5 mg/kg	1.269

Group A	Group B	Calculated t-value
Atrazine (Water rinsing) 0.5 mg/kg	Terbuthylazine (Water rinsing) 0.5 mg/kg	0.084
Atrazine (Water rinsing) 0.5 mg/kg	Acetochlor (Water rinsing) 0.5 mg/kg	**8.171**
Atrazine (Water rinsing) 0.5 mg/kg	Alachlor (Water rinsing) 0.5 mg/kg	**8.842**
Terbuthylazine (Water rinsing) 0.5 mg/kg	Acetochlor (Water rinsing) 0.5 mg/kg	**9.458**
Terbuthylazine (Water rinsing) 0.5 mg/kg	Alachlor (Water rinsing) 0.5 mg/kg	**10.336**
Acetochlor (Water rinsing) 0.5 mg/kg	Alachlor (Water rinsing) 0.5 mg/kg	1.483
Atrazine (Acetonitrile & NaCl) 0.5 mg/kg	Atrazine (Water rinsing) 0.5 mg/kg	2.555
Terbuthylazine (Acetonitrile & NaCl) 0.5 mg/kg	Terbuthylazine (Water rinsing) 0.5 mg/kg	**3.173**
Acetochlor (Acetonitrile & NaCl) 0.5 mg/kg	Acetochlor (Water rinsing) 0.5 mg/kg	2.036
Alachlor (Acetonitrile & NaCl) 0.5 mg/kg	Alachlor (Water rinsing) 0.5 mg/kg	2.148

Table 4.58 was divided into three sections. The top section represents the 0.05 mg/kg spiked cannabis sample NISPE experiments. Since no acetochlor or alachlor was detected, only atrazine and terbuthylazine were compared and no statistical differences were found. The results from the t-test strongly indicate that the NIP has a higher affinity for the triazine pesticides. This can once again be correlated to the hypothesis that the triazines have more opportunity for hydrogen bonding than chloroacetamide pesticides, as depicted in Section 2.7.2.3.

In the second section of Table 4.58, 0.5 mg/kg spiked cannabis NISPE experiments were compared. A statistical difference was found every time a triazine pesticide was compared to a chloroacetamide pesticide. This again indicates the higher affinity or stronger hydrogen bonding of the triazine pesticides to the NIP sorbent. The last section of Table 4.58, where the acetonitrile and water extraction methods were compared, only terbuthylazine was found to have a statistical difference between the two extraction methods, unlike the terbuthylazine NISPE experiments at the lower concentration where no statistical difference was observed.

Comparison to literature

A similar NISPE was used for the extraction of atrazine from environmental water samples. The recovery was found to be only 24% with a %RSD of 1% for a mL water sample that was spiked at 0.8 µg/L with atrazine. In the same experiment, alachlor was reported to have a 2% recovery with a %RSD of 0.1% on the same NIP (Kueseng *et al.*, 2009). The spiking concentration of 0.8 µg/L was far lower than the 50 µg/kg of this experiment with a much larger loading fraction of 500 mL employed. As in this literature example, a much lower recovery was observed for the chloroacetamide pesticides on the NIP in this study.

For a similar NIP, prepared using the same monomers but using dichloromethane as porogen, a 26% recovery was reported for terbuthylazine extracted from a 50 mL 1 µg/L spiked ground water sample (Ferrer *et al.*, 2000). No recovery was reported for atrazine on the same NIP at 1 µg/L concentration. A NIP more similar to the one in this study (with toluene as porogen) was also tested for both atrazine and terbuthylazine recovery at 1 µg/L, but a 0% recovery was reported for both analytes. When the loading fraction was doubled (100 mL 1 µg/L spiked ground water sample) 5 and 6% recoveries were reported for atrazine and terbuthylazine on the toluene porogen NIP (Ferrer *et al.*, 2000). It can thus be deduced that the more analyte that was passed through the NISPE, the higher the observed recoveries. The same observation can be made from Table 4.57; when the concentration of analyte was increased, the recovery of each analyte also increased.

Another similar NIP was reported by Guzella et al. and used for the extraction of atrazine and terbuthylazine from spiked river water at a much higher concentration (5 mg/L or 5 µg/mL). The recoveries reported were 7.2 and 7.7% for atrazine and terbuthylazine, respectively. The wash fraction employed was dichloromethane, and the % loss reported in the wash fraction was 64.6 and 62.5% for atrazine and terbuthylazine, respectively (Guzzella *et al.,* 2008). The high loss in the organic wash solvent does indicate that atrazine and terbuthylazine were both trapped on the NISPE sorbent during the loading step, suggesting that the NIP does have some affinity for these pesticides. Taking the sum of the elution fraction and wash fraction concentrations from the literature example indicates at least 71.8 and 70.2% of each respective pesticide was trapped to the sorbent during the loading fraction. These values are comparable to the higher 0.5 mg/kg experiments performed in this study (Table 4.57).

4.7.4: Recovery of extracted triazine pesticide spiked onto cannabis flowers from triazine MISPEs

Table 4.59 and 4.60 display the results obtained for spiked cannabis flower samples extracted utilising the methodology as discussed in Section 3.8 with clean-up using the atrazine and terbuthylazine MISPEs respectively. Prior to MISPE, the 2 mL conditioning methanol was analysed utilising GC-MS to ensure no template was eluting from the sorbent. Replicates 1 to 3 represent samples extracted using the acetonitrile & NaCl methodology that was reconstituted with 150 µL methanol containing 5 ppm caffeine IS resulting in calculated theoretical concentrations of around 1.00 µg/mL post reconstitution. The dried elution fraction would have been able to dissolve in less reconstitution solvent, however, to ensure comparability to the C18 SPE experiments, the same reconstitution volume was used.

Table 4.59: Result summary for atrazine MISPE experiments performed from cannabis plant material

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
1	Atrazine	Acetonitrile & NaCl	1.00	**0.70**	70.3	4.8	75.8	6.3
2	Atrazine	Acetonitrile & NaCl	1.00	**0.75**	75.2			
3	Atrazine	Acetonitrile & NaCl	0.98	**0.81**	82.0			
1	Terbuthylazine	Acetonitrile & NaCl	1.00	**0.82**	82.5	4.0	78.1	5.1
2	Terbuthylazine	Acetonitrile & NaCl	1.00	**0.73**	72.8			
3	Terbuthylazine	Acetonitrile & NaCl	0.98	**0.78**	79.0			
4	Atrazine	Water rinsing	2.99	**1.62**	54.0	3.9	58.5	6.7
5	Atrazine	Water rinsing	2.97	**1.72**	58.0			
6	Atrazine	Water rinsing	3.04	**1.93**	63.5			
4	Terbuthylazine	Water rinsing	2.99	**1.68**	56.2	4.6	60.1	7.7
5	Terbuthylazine	Water rinsing	2.97	**1.98**	66.6			
6	Terbuthylazine	Water rinsing	3.04	**1.75**	57.6			

Table 4.60: Result summary for terbuthylazine MISPE experiments performed from cannabis plant material

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
1	Atrazine	Acetonitrile & NaCl	1.01	**0.81**	80.6	3.5	76.4	4.5
2	Atrazine	Acetonitrile & NaCl	0.99	**0.75**	76.6			
3	Atrazine	Acetonitrile & NaCl	0.98	**0.70**	72.1			
1	Terbuthylazine	Acetonitrile & NaCl	1.01	**0.76**	75.0	3.7	77.1	4.8
2	Terbuthylazine	Acetonitrile & NaCl	0.99	**0.81**	82.4			
3	Terbuthylazine	Acetonitrile & NaCl	0.98	**0.72**	74.1			

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
4	Atrazine	Water rinsing	2.95	**1.97**	66.9	3.0	63.8	4.7
5	Atrazine	Water rinsing	3.04	**1.82**	59.8			
6	Atrazine	Water rinsing	2.92	**1.89**	64.7			
4	Terbuthylazine	Water rinsing	2.95	**1.82**	61.7	3.0	59.9	5.1
5	Terbuthylazine	Water rinsing	3.04	**1.89**	62.3			
6	Terbuthylazine	Water rinsing	2.92	**1.62**	55.6			

From the data in Table 4.59 and 4.60, the standard deviation and mean recovery values were calculated and t-tests were performed to compare the different triplicate results. The t_{crit} value was identified as 2.776 at a 95% confidence interval and 4 degrees of freedom. The t-test results are presented in Table 4.61.

Table 4.61: T-tests for the recoveries of triazine pesticides extracted from spiked cannabis flower samples with triazine MISPE clean-up

Group A recoveries	Group B recoveries	Calculated t-value
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	0.735
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	Atrazine (Water rinsing) 0.05mg/kg on atrazine MISPE.	**5.609**
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	Terbuthylazine (Water rinsing) 0.05mg/kg on atrazine MISPE.	**4.711**
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	Atrazine (Water rinsing) 0.05mg/kg on atrazine MISPE.	**7.004**
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	Terbuthylazine (Water rinsing) 0.05mg/kg on atrazine MISPE.	**5.864**
Atrazine (Water rinsing) 0.05mg/kg on atrazine MISPE	Terbuthylazine (Water rinsing) 0.05mg/kg on atrazine MISPE.	0.531
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on terbuthylazine MISPE	Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/ on terbuthylazine MISPE	0.287
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on terbuthylazine MISPE	Atrazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	**5.513**
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on terbuthylazine MISPE	Terbuthylazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	**7.194**
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on terbuthylazine MISPE	Atrazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	**5.599**
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on terbuthylazine MISPE	Terbuthylazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	**7.216**

Group A recoveries	Group B recoveries	Calculated t-value
Atrazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	Terbuthylazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	1.861
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on terbuthylazine MISPE	0.198
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on atrazine MISPE	Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on terbuthylazine MISPE	0.361
Atrazine (Water rinsing) 0.05mg/kg on atrazine MISPE	Atrazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	2.163
Terbuthylazine (Water rinsing) 0.05mg/kg on atrazine MISPE.	Terbuthylazine (Water rinsing) 0.05mg/kg on terbuthylazine MISPE	0.093

The first and second sections of Table 4.61 represent the t-tests within the atrazine MISPE and terbuthylazine MISPE groups, respectively. A clear statistical difference was found between the acetonitrile & NaCl and water rinsing extraction methods. The water rinsing method was found to be far less effective for the extraction of the triazine pesticides from the cannabis plant material. This can be attributed to the low water solubility of atrazine and terbuthylazine as discussed in Section 2.2.4. No statistical differences were observed when comparing atrazine and terbuthylazine recoveries on either the atrazine or terbuthylazine MISPEs. The third section of Table 4.61 represents an inter-MIP comparison, where the recoveries relate to atrazine and terbuthylazine extracted with the same methods but with differently imprinted MISPEs. The templates were thus found to be compatible for both MIPs, as no statistical differences were observed in this third section of Table 4.61. This cross compatibility has been observed in literature as well between atrazine and terbuthylazine on a terbuthylazine MIP (Ferrer *et al.*, 2000).

Comparison to literature

A study utilising a similar atrazine MIP, reported recoveries of 94 to 99% with %RSDs < 2% from atrazine spiked environmental water samples. The LOD was reported as 0.08 µg/L water (Kueseng *et al.*, 2009). This was almost a thousand-fold lower than the 0.05 mg/kg MRL for atrazine on South African crops, however it can be argued that water was a far less complex matrix and requires minimal workup before sample extraction can be performed.

In another literature example, two similar terbuthylazine imprinted MIPs were prepared and compared using different porogens (toluene and dichloromethane). MISPE recoveries, for atrazine and terbuthylazine, ranging from 83 to 96% were reported for spiked groundwater samples (1 µg/L). This concentration was lower than that of the spiked cannabis flowers, 1 ppb vs 50 ppb respectively. It was reported that atrazine had a better recovery of 91% on the dichloromethane porogen MIP compared to 83% on the toluene MIP. The results for terbuthylazine were found to be reversed, in

that 96% recovery was reported on the toluene porogen MIP and 84% on the dichloromethane porogen MIP. When the loading fraction was doubled (100 mL 1 µg/L spiked groundwater sample), 82 and 96% recoveries were reported for atrazine and terbuthylazine respectively on the toluene porogen MIP. The use of dichloromethane as wash solvent resulted in recoveries of under 60% for both triazine pesticides on the MISPEs (Ferrer *et al.*, 2000).

Another study, used a dummy template (propazine) MIP with the same monomers, activator and porogen as in this study, for the extraction of atrazine and terbuthylazine from spiked river water at a much higher concentration (5 mg/L or 5 µg/mL). Recoveries of 74.6 and 68.5% were reported for atrazine and terbuthylazine respectively (Guzzella *et al.,* 2008). The MISPE recoveries reported by Guzzella *et al.* were relatively low compared to the previously mentioned literature examples, especially considering that the matrix was river water and the spiked concentration was 100x higher (5 ppm vs 0.05 ppm) than in this study. The reason for the relatively low recoveries might be attributed to the wash solvent (dichloromethane) used during the MISPE procedure. Using an organic wash solvent removed much of the analyte too early during the extraction procedure. The use of dummy templates might also have reduced the affinity of the MIP for the target pesticides.

A more relevant literature example might be the use of a triazine (propazine) MISPE on a TiO_2 surface for the extraction of three triazine pesticides, including atrazine, from maize. Recoveries of 82-99% were reported at a spiked concentration as low as 1 µg/kg (Geng *et al.*, 2015), which was significantly higher than that obtained in this study.

Comparison to the C18 SPE

One of the aims of this study was to compare the synthesised MIPs to a commercially available C18 sorbent cartridge. Although the recoveries for both atrazine and terbuthylazine were observed to be lower from the atrazine and terbuthylazine MISPEs compared to the C18 SPE, it could be observed both visually (from the colour of the eluate) and chromatographically that the synthesised MIPs were far more selective to their template pesticides than the C18 sorbent.

The following terbuthylazine chromatograms (Figure 4.63 and 4.64) were selected to illustrate the large difference in selectivity observed between the C18 and terbuthylazine MIP sorbents. Both samples were extracted from +- 3 g cannabis flower spiked at a concentration of 0.05 mg/kg terbuthylazine and extracted using the acetonitrile & NaCl method as described in Section 3.8.2. Figure 4.63 shows the chromatogram for terbuthylazine (*m/z* 214) after C18 SPE was performed. Figure 4.64 displays the equivalent chromatogram after terbuthylazine MISPE was performed. There were clearly many more compounds trapped and subsequently eluted from the C18 sorbent compared to the terbuthylazine MISPE. The fewer interferences in the chromatogram enhances the LOD as the signal

to noise was increased for the peak in question. The instrument maintenance can also be also expected to decrease as the liner will stay cleaner for longer and the column lifetime will be increased as less matrix interferences will interact with the stationary phase.

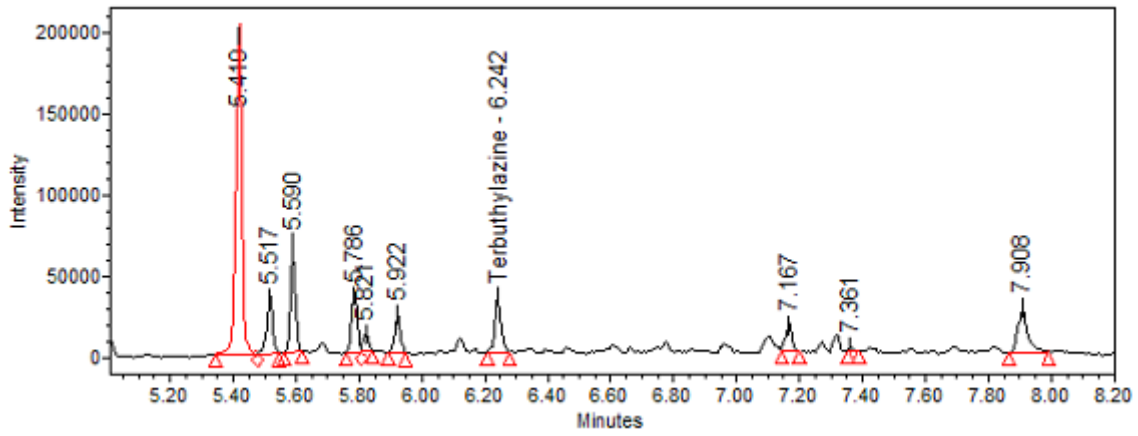


Figure 4.63: SIR chromatogram for terbuthylazine (m/z 214) after C18 SPE was performed on a +- 3 g cannabis flower spiked at a concentration of 0.05 mg/kg terbuthylazine

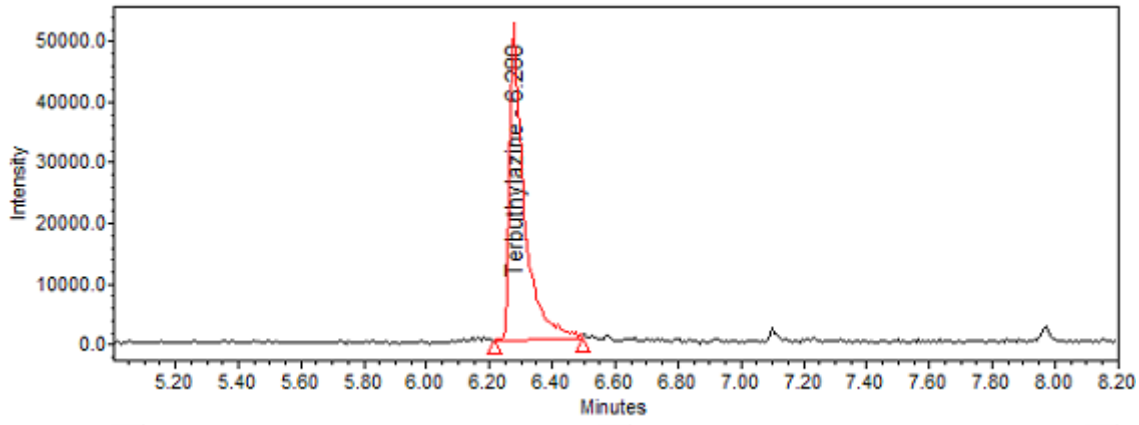


Figure 4.64: SIR chromatogram for terbuthylazine (m/z 214) after terbuthylazine MISPE was performed on a +- 3 g cannabis flower spiked at a concentration of 0.05 mg/kg terbuthylazine

4.7.5: Recovery of extracted chloroacetamide pesticides spiked onto cannabis flowers from chloroacetamide MISPEs

Table 4.62 and 4.63 displays the results obtained for spiked cannabis flower samples extracted utilising the methodology as discussed in Section 3.8 on the acetochlor and alachlor MISPEs respectively. Prior to MISPE, the 2 mL conditioning methanol was analysed utilising GC-MS to ensure no template was eluting from the sorbent. Replicates 1 to 3 represent samples extracted using the acetonitrile & NaCl methodology that was reconstituted with 150 µL methanol containing 5 ppm caffeine IS resulting in calculated theoretical concentrations of around 1.00 µg/mL post reconstitution. The dried elution

fraction would have been able to dissolve in less reconstitution solvent, however, to ensure comparability to the C18 SPE experiments the same reconstitution volume was used.

Table 4.62: Result summary for acetochlor MISPE experiments performed from cannabis plant material

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
1	Acetochlor	Acetonitrile & NaCl	1.03	**0.68**	66.2	4.3	**60.2**	7.1
2	Acetochlor	Acetonitrile & NaCl	0.97	**0.55**	56.6			
3	Acetochlor	Acetonitrile & NaCl	1.02	**0.59**	57.8			
1	Alachlor	Acetonitrile & NaCl	1.03	**0.71**	69.1	6.4	**60.8**	10.5
2	Alachlor	Acetonitrile & NaCl	0.97	**0.52**	53.5			
3	Alachlor	Acetonitrile & NaCl	1.02	**0.61**	59.7			
4	Acetochlor	Water rinsing	2.96	**2.43**	82.2	8.3	**79.3**	10.5
5	Acetochlor	Water rinsing	3.06	**2.69**	87.8			
6	Acetochlor	Water rinsing	3.07	**2.09**	68.0			
4	Alachlor	Water rinsing	2.96	**2.72**	92.0	9.5	**79.6**	11.9
5	Alachlor	Water rinsing	3.06	**2.38**	77.7			
6	Alachlor	Water rinsing	3.07	**2.12**	69.0			

Table 4.63: Result summary for alachlor MISPE experiments performed from cannabis plant material

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
1	Acetochlor	Acetonitrile & NaCl	0.99	**0.69**	69.5	7.5	**61.5**	12.2
2	Acetochlor	Acetonitrile & NaCl	1.01	**0.52**	51.4			
3	Acetochlor	Acetonitrile & NaCl	0.99	**0.63**	63.6			
1	Alachlor	Acetonitrile & NaCl	0.99	**0.70**	70.5	6.2	**62.1**	10.0
2	Alachlor	Acetonitrile & NaCl	1.01	**0.61**	60.3			
3	Alachlor	Acetonitrile & NaCl	0.99	**0.55**	55.6			
4	Acetochlor	Water rinsing	2.92	**2.45**	84.0	8.6	**85.0**	10.2
5	Acetochlor	Water rinsing	2.93	**2.81**	96.0			
6	Acetochlor	Water rinsing	2.96	**2.22**	74.9			
4	Alachlor	Water rinsing	2.92	**2.36**	80.9	9.5	**83.8**	11.4
5	Alachlor	Water rinsing	2.93	**2.83**	96.7			
6	Alachlor	Water rinsing	2.96	**2.19**	73.9			

From the data in Table 4.62 and 4.63 the standard deviation and mean recoveries values were calculated and t-tests were performed to compare the different triplicate results. The t_{crit} value was

identified as 2.776 at a 95% confidence interval and 4 degrees of freedom. The t-test results are presented in Table 4.64.

Table 4.64: T-tests for the recoveries of triazine pesticides extracted with triazine MISPEs from spiked cannabis flower samples

Group A recoveries	Group B recoveries	Calculated t-value
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE	Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE	0.155
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE	Acetochlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	**4.094**
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE	Alachlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	**3.729**
Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE	Acetochlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	**3.532**
Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE.	Alachlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	**3.283**
Alachlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	Acetochlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	0.035
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE	Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE	0.125
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE	Acetochlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	**4.099**
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE	Alachlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	**3.680**
Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE	Acetochlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	**4.294**
Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE.	Alachlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	**3.817**
Alachlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	Acetochlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	0.177
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE	Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE	0.306
Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on acetochlor MISPE	Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on alachlor MISPE	0.299
Acetochlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	Acetochlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	0.939
Alachlor (Water rinsing) 0.05 mg/kg on acetochlor MISPE	Alachlor (Water rinsing) 0.05 mg/kg on alachlor MISPE	0.636

The first and second sections of Table 4.64 represent the t-tests within the acetochlor MISPE and alachlor MISPE groups respectively. A clear statistical difference was found between the acetonitrile & NaCl and water rinsing extraction methods. The water rinsing method was found to be more effective for the extraction of the chloroacetamide pesticides from the cannabis plant material. This can be attributed to the higher water solubility of acetochlor and alachlor as discussed in Section 2.2.4.

No statistical differences were observed when comparing acetochlor and alachlor recoveries on either the acetochlor or alachlor MISPEs. The third section of Table 4.64 represents an inter-MIP comparison, where the recoveries of acetochlor and alachlor extracted with the same methods but with differently imprinted MISPEs. The templates were thus found to be compatible for both pesticides, as no statistical differences were observed in this third section of Table 4.64. The use of dummy templates in literature supports the cross compatibility as observed, as both acetochlor and alachlor could be extracted utilising the same MIP (Wang *et al.*, 2015).

Comparison to literature

There are remarkably few literature articles for chloroacetamide pesticide MIPs and particularly MISPE applications when compared to the previously discussed triazine pesticides.

The mean recoveries of four chloroacetamide herbicides, including alachlor and acetochlor, extracted with a similar dummy template MIP (same monomers) from blank soil spiked at a range of concentrations from 0.1 to 1 µg/g ranged between 80.6 and 90.2% with %RSDs of under 8% (n =5). The LOD was reported at 0.0005 to 0.025 mg/kg for the chloroacetamide herbicides (Wang *et al.*, 2015). This was, however, lower than the 0.05 mg/kg MRL for acetochlor and alachlor on South African crops, although the matrix was of course less complex than the cannabis flower matrix in terms of organic compounds present.

Comparison to C18 SPE

The recoveries for both acetochlor and alachlor were observed to be lower from the acetochlor and alachlor MISPEs compared to the C18 SPE, however it could be both visually (from the colour of the eluate) and chromatographically observed that the synthesised MIPs were far more selective to their template pesticides than the C18 sorbent.

The following alachlor chromatograms (Figure 4.65 and 4.66) were selected to illustrate the difference in selectivity observed between the C18 and acetochlor MIP sorbents. Both samples were extracted from +- 3 g cannabis flower spiked at a concentration of 0.05 mg/kg alachlor and extracted using the water rinsing method as described in Section 3.8.2. Figure 4.65 shows the chromatogram for alachlor (*m/z* of 188) after C18 SPE was performed. Figure 4.66 displays the equivalent chromatogram after acetochlor MISPE was performed. The matrix interferences in both chromatograms are similar, although after the MISPE, the alachlor was clearly the major component in the chromatogram and after C18 the equivalent peak was dwarfed by the matrix interferences.

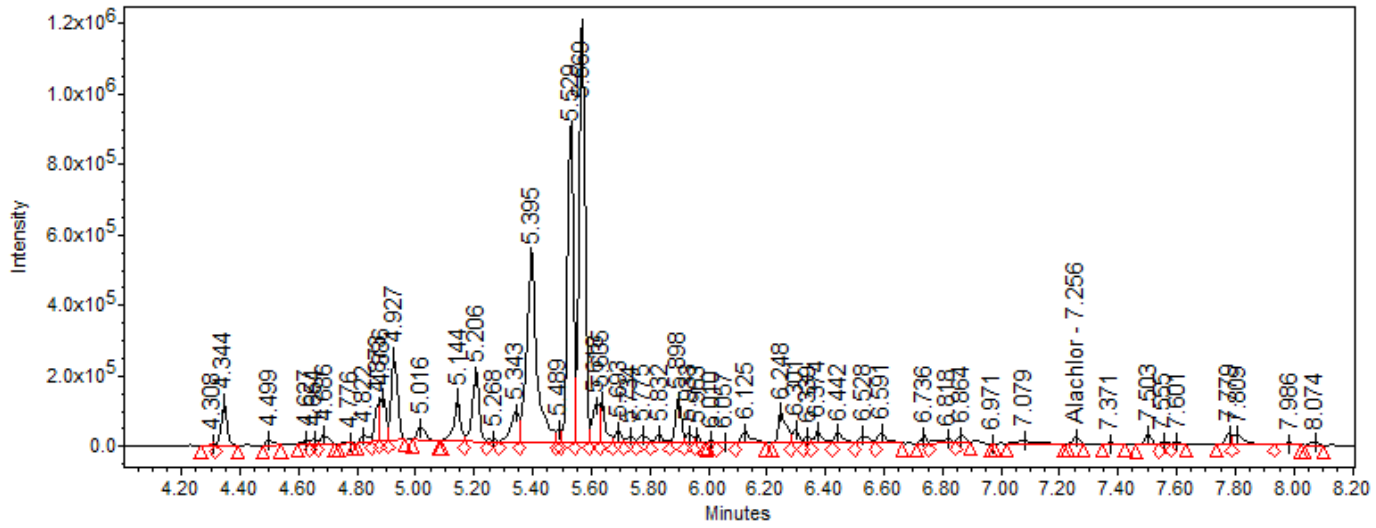


Figure 4.65: SIR chromatogram for alachlor (m/z 188) after C18 SPE was performed on a +- 3 g cannabis flower spiked at a concentration of 0.05 mg/kg alachlor

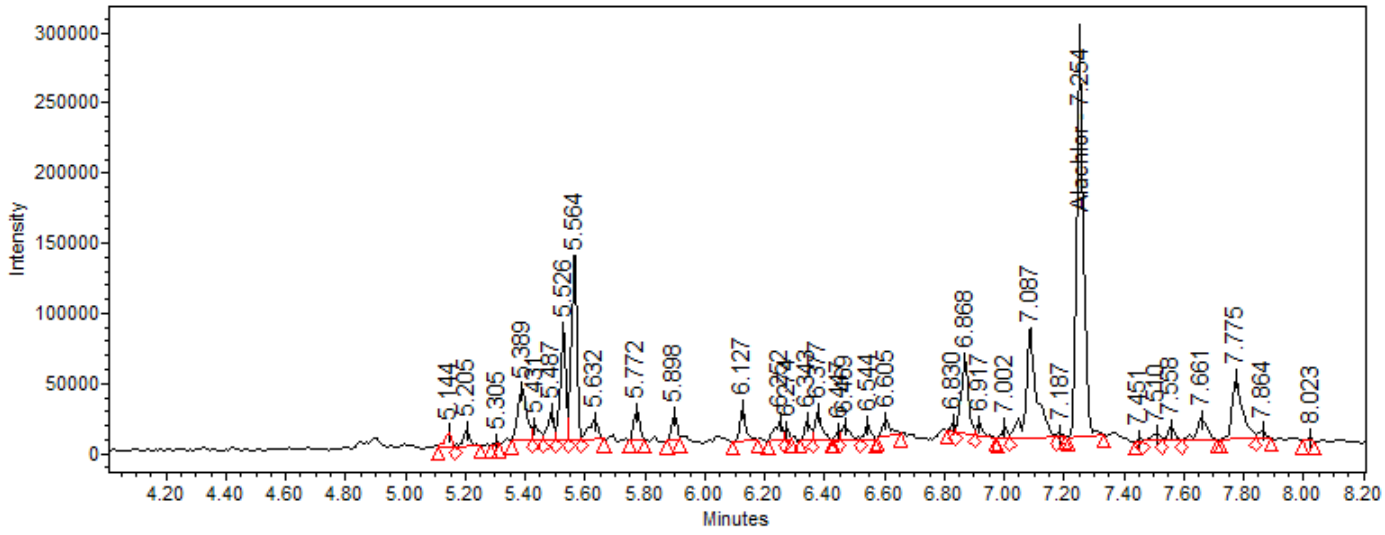


Figure 4.66: SIR chromatogram for alachlor (m/z 188) after alachlor MISPE was performed on a +- 3 g cannabis flower spiked at a concentration of 0.05 mg/kg alachlor

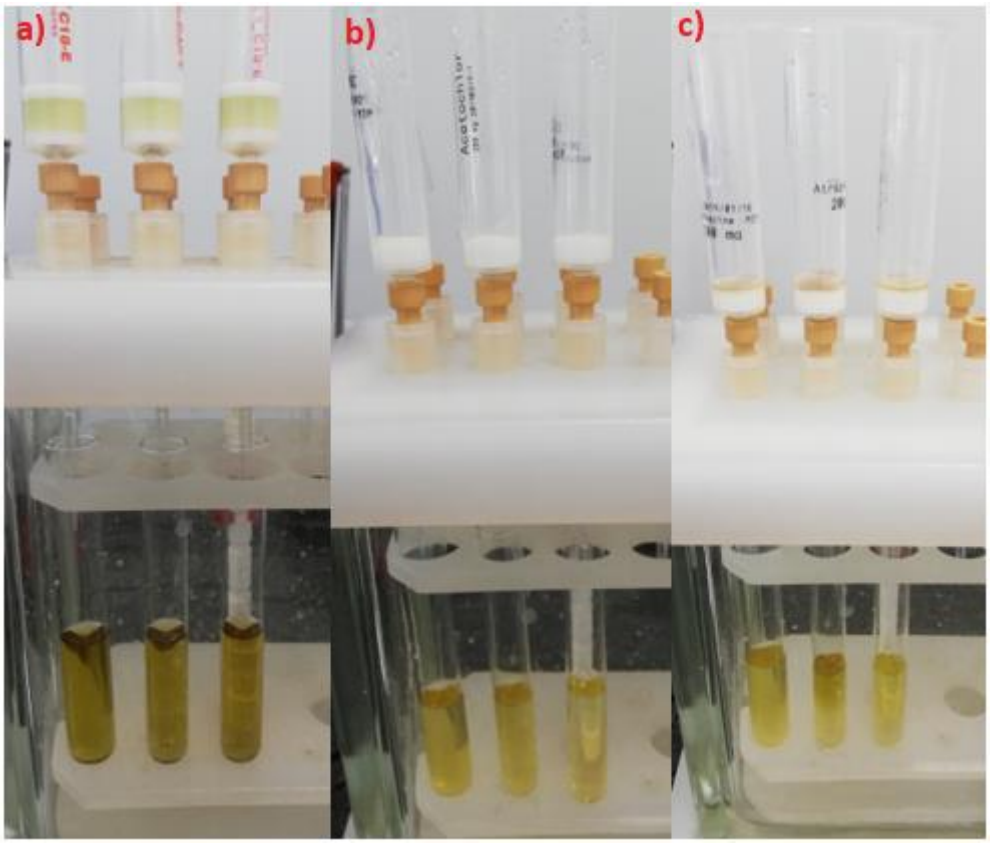


Figure 4.67: Visual comparison of the methanol elution fractions from 3 g extracted cannabis flower samples. A) represents the C18 SPE cartridges, b) represents acetochlor MISPE cartridges and c) represents atrazine MISPE cartridges

From Figure 4.67 it can be seen that the C18 sorbent was discoloured by matrix compounds that were retained from the cannabis flowers, where neither the acetochlor or atrazine MIP sorbent were discoloured. The methanol eluent of both MISPEs (b and c) are clearer and less discoloured than the eluent of the C18 SPE (a). Samples were observed to be cleaner when atrazine or terbuthylazine was extracted because higher concentrations of methanol were used for the washing steps when compared to acetochlor and alachlor extractions with very little methanol added. All the MISPEs yielded noticeably clearer eluates than the C18 SPE samples.

4.7.6: Recovery of both extracted triazine and chloroacetamide pesticides spiked onto cannabis flowers from the multi template MISPE

Table 4.65 display the results obtained for spiked cannabis flower samples extracted utilising the methodology as discussed in Section 3.8 on the MTMISPEs. Prior to MISPE, the 2 mL conditioning methanol was analysed utilising GC-MS to ensure no template was eluting from the sorbent. Replicates 1 to 3 represent samples extracted using the acetonitrile & NaCl methodology that were reconstituted with 150 µL methanol containing 5 ppm caffeine IS resulting in calculated theoretical

concentrations of around 1 µg/mL post reconstitution. Replicates 4 to 6 represent the water rinsing experiments, where combined aqueous solutions were passed through the MTMISPE.

Table 4.65: Result summary for the multi template MISPE experiments performed from cannabis plant material

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
1	Atrazine	Acetonitrile & NaCl	1.01	**0.76**	75.2	4.3	**76.5**	5.6
2	Atrazine	Acetonitrile & NaCl	1.01	**0.83**	82.3			
3	Atrazine	Acetonitrile & NaCl	0.97	**0.70**	71.9			
1	Terbuthylazine	Acetonitrile & NaCl	1.01	**0.87**	86.1	5.0	**81.2**	6.2
2	Terbuthylazine	Acetonitrile & NaCl	1.01	**0.75**	74.3			
3	Terbuthylazine	Acetonitrile & NaCl	0.97	**0.81**	83.2			
1	Acetochlor	Acetonitrile & NaCl	1.01	**0.58**	57.4	5.7	**59.9**	9.5
2	Acetochlor	Acetonitrile & NaCl	1.01	**0.55**	54.5			
3	Acetochlor	Acetonitrile & NaCl	0.97	**0.66**	67.8			
1	Alachlor	Acetonitrile & NaCl	1.01	**0.69**	68.3	3.6	**63.4**	5.7
2	Alachlor	Acetonitrile & NaCl	1.01	**0.63**	62.4			
3	Alachlor	Acetonitrile & NaCl	0.97	**0.58**	59.6			
4	Atrazine	Water rinsing	3.08	**1.54**	50.1	6.8	**58.9**	11.5
5	Atrazine	Water rinsing	3.05	**1.83**	60.0			
6	Atrazine	Water rinsing	2.90	**1.93**	66.5			
4	Terbuthylazine	Water rinsing	3.08	**1.84**	59.8	3.3	**59.9**	5.5
5	Terbuthylazine	Water rinsing	3.05	**1.95**	63.9			
6	Terbuthylazine	Water rinsing	2.90	**1.62**	55.8			
4	Acetochlor	Water rinsing	3.08	**2.71**	88.1	5.8	**83.2**	7.0
5	Acetochlor	Water rinsing	3.05	**2.29**	75.1			
6	Acetochlor	Water rinsing	2.90	**2.51**	86.5			

Rep. (#)	Pesticide	Extraction method	Calculated theoretical concentration after reconstitution (µg/mL)	Determined concentration by GC-MS (µg/mL)	Recovery (%)	STDEV (%)	Mean recovery (%)	RSD (%)
4	Alachlor	Water rinsing	3.08	**2.54**	82.6	4.9	**81.7**	6.0
5	Alachlor	Water rinsing	3.05	**2.30**	75.4			
6	Alachlor	Water rinsing	2.90	**2.53**	87.2			

From the data in Table 4.65 the standard deviation and mean recovery values were calculated and t-tests were performed to compare the different triplicate results. The t_{crit} value was identified as 2.776 at a 95% confidence interval and 4 degrees of freedom (n_1+n_2-2). The t-test results are presented in Table 4.66.

Table 4.66: T-tests for the recoveries of pesticides cleaned up from the extract with the MT MISPEs from spiked cannabis flower samples

Group A recoveries	Group B recoveries	Calculated t-value
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	1.438
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	**4.628**
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	**4.622**
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	**5.612**
Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	**5.750**
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	1.045
Atrazine (Water rinsing) 0.05 mg/kg on MTMISPE	Terbuthylazine (Water rinsing) 0.05 mg/kg on MTMISPE	0.265
Atrazine (Water rinsing) 0.05 mg/kg on MTMISPE	Acetochlor (Water rinsing) 0.05 mg/kg on MTMISPE	**5.460**
Atrazine (Water rinsing) 0.05 mg/kg on MTMISPE	Alachlor (Water rinsing) 0.05 mg/kg on MTMISPE	**5.481**
Terbuthylazine (Water rinsing) 0.05 mg/kg on MTMISPE	Acetochlor (Water rinsing) 0.05 mg/kg on MTMISPE	**6.998**
Terbuthylazine (Water rinsing) 0.05 mg/kg on MTMISPE	Alachlor (Water rinsing) 0.05 mg/kg on MTMISPE	**7.440**
Acetochlor (Water rinsing) 0.05 mg/kg on MTMISPE	Alachlor (Water rinsing) 0.05 mg/kg on MTMISPE	0.396
Atrazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Atrazine (Water rinsing) 0.05 mg/kg on MTMISPE	**4.384**

Terbuthylazine (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Terbuthylazine (Water rinsing) 0.05 mg/kg on MTMISPE	**7.126**
Acetochlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Acetochlor (Water rinsing) 0.05 mg/kg on MTMISPE	**5.726**
Alachlor (Acetonitrile & NaCl) 0.05 mg/kg on MTMISPE	Alachlor (Water rinsing) 0.05 mg/kg on MTMISPE	**6.023**

As observed with the previous MISPE experiments in Sections 4.7.5 and 4.7.6, Table 4.66 shows the acetonitrile & NaCl extraction of spiked cannabis plant material to be more effective for the triazine pesticides and the water rinsing extraction method more effective for the chloroacetamide pesticides. No significant differences were observed between the two triazine pesticides, nor between the two chloroacetamide pesticides.

Chapter 5 : Conclusions and future work

The overall conclusion of this study is based on the extraction efficiency, selectivity, cost and potential waste of various MIPs in comparison to the NIP and C18 reference sorbents, particularly with regards to the matrix of interest. In terms of extraction efficiency, both the synthesised MIPs and the C18 sorbent employed proved to be efficient at extracting the pesticides from spiked cannabis plant material. Only the NIP proved ineffective as the 0.05 mg/kg limit could not be reached. The MIPs proved to be very selective for their template class pesticide, as was evident from the chromatograms after the C18 SPE and MISPE procedures were used for the extraction on spiked cannabis samples. The C18 SPEs employed were found to be more cost effective, especially when labour was considered. However, instrument operating costs can be expected to be lower when the MIP is employed, because more matrix interferences can be removed with the MIP. In terms of environmental considerations, the only real drawback of the MIPs was found to be the use of concentrated high purity template pesticide during synthesis. Proper waste management cannot be stressed enough when MIPs are produced, as the pesticide templates used in synthesis can pose a serious threat to the environment and human health.

The MIPs had far greater selectivity, as they target a specific pesticide class depending on the template used during synthesis, whereas the C18 sorbent traps and subsequently co-elutes many nonpolar molecules present in the cannabis plant material. The lack of selectivity of the C18 sorbent results in chromatograms containing many additional peaks on the SIR mass channels used for quantitation of the target analytes, leading to reduced sensitivity. The MIPs are far more labour-intensive in terms of SPE and sample preparation. Polymerization, grinding, sieving, and packing of the MISPE obviously takes longer than buying the commercially available pre-packed C18 SPE cartridges. Additionally, the packed sorbent bed was observed to damage easily when the sorbent dried or too much vacuum was applied, resulting in samples having to be re-run because of low recoveries. However, the argument can be made that the MISPEs reduce labour costs relating to instrument maintenance and are potentially cost saving with respect to the GC-MS instrumentation. Since the MIP sorbents were demonstrated to have greater selectivity than the C18 sorbent during the extraction procedure, fewer foreign compounds are injected onto the GC-MS. This will result in the inlet liner staying cleaner for longer and could potentially lengthen the column, filament and multiplier lifetimes.

5.1: Interactions of the target pesticides with the synthesised polymers

Hydrogen bonding was theorized to be the fundamental interaction between the MIP and the pesticide template. It was demonstrated in the wash solvent optimization experiments and in the recovery determination experiment that the NIP sorbent has a higher affinity for the triazine pesticides than the chloroacetamide pesticides. This is likely because there are more opportunities for unoriented or random hydrogen bonding to occur with the functional groups of the sorbent to the analyte. These are clearly not the only interactions to consider, as the NIP adsorption capacity experiments revealed a correlation between adsorption capacity and the log K_{OW} values of the pesticides. There were experimental differences between these experiments, the most obvious of which is that the NIP was packed into a SPE cartridge with flowing loading, wash and elution solvent in the one set of experiments as compared to a stationary experiment in the determination of the adsorption capacity. In the recovery experiments from spiked cannabis samples, the spiked pesticides were also nowhere near the maximum adsorption capacity of the sorbent, surely making it less of a factor to consider.

It can be theorised that the number of hydrogen bonds between the sorbent and analyte is an important factor for the retention of the pesticides on the NIP or MIP sorbent under the flow conditions of the SPE procedure. This was demonstrated by the oriented and multiple hydrogen bonds in the MIP cavities having a stronger affinity for the template than the NIP with its random hydrogen bonds, and each of the MIPs had higher recoveries for their templates compared to the NIP.

It may have occurred in some instances that the random hydrogen bonds in the NIP could potentially bind to a pesticide from more than one functional unit in the polymer, resulting in the pesticide analyte having more than one interaction with the sorbent. The potential hydrogen bonds are discussed in Section 2.7.2.3. It was hypostasized that there are more hydrogen bonding sites for atrazine and terbuthylazine, with five amines, than for acetochlor and alachlor with a maximum of three hydrogen bonding sites with an amide and ether functional groups. The combination of the aforementioned factors might explain why the NISPE proved to have better recoveries for the triazine pesticides, as multiple interactions to the triazine was simply more likely compared to the chloroacetamide pesticides.

Experimentally observed differences between triazine and chloroacetamide pesticides during the MISPE and C18 experiments.

The acetonitrile & NaCl method was found to be more effective for the extraction of triazine pesticides, whilst the water rinsing method was more effective for chloroacetamide pesticides. This can largely be attributed to acetochlor and alachlor having higher water solubilities than atrazine and terbuthylazine. However, in the wash solvent optimization experiments, more organic solvent was required to elute acetochlor and alachlor from the C18 sorbent compared to the triazine pesticides. This may be explained by the log K_{ow} values, except that almost paradoxically both triazine pesticides eluted before the chloroacetamide pesticides. Then, on the MISPEs and NISPE, acetochlor and alachlor always eluted before terbuthylazine and atrazine, thus with less organic solvent. This is a clear indication that the NISPE and MISPEs have different interactions with the analytes leading to their retention. This proves that the MIP retains template analytes due to orientated hydrogen bonding and the C18 largely through hydrophobic interactions.

The elution order on both the C18 SPE and MISPEs were not found to correlate to the adsorption capacity, which was likely due to the different experimental conditions, where SPE was performed under flow conditions and the adsorption capacity was determined under stationary conditions.

5.2: Stability of the synthesised polymers

Since this study was conducted over several years, often a polymer that was washed and sieved was stored for months before being packed into SPE cartridges and it still performed well. The integrity of the packed cartridges is rather what decays; it was found to be imperative that they were stored wet, as visible cracks and channels form in the polymer packing once it dried.

This channelling happened quickly and could be observed when the cartridge was dried before elution in the SPE method. The drying step was thus omitted from the MISPE and NISPE methods. This was not observed for the purchased C18 cartridges, indicating either the C18 sorbent is less prone to channelling or the commercial packing and compression of the cartridges were more optimal.

5.3: Waste and environmental effects

During MIP synthesis, a pesticide template was used and subsequently washed out. The wet sieving process also produces contaminated waste. Atrazine is a major pollutant of soil and water ecosystems (Singh *et al.*, 2018). The EPA limit for atrazine in drinking water is 3 µg/L (Kueseng *et al.*, 2009). The

small-scale MIP synthesis used just over 5 mg of atrazine, and considering the EPA limit, 5000 µg/ 3 µg/L = 1666.6 l. This means one small scale synthesis has the potential to contaminate a large volume of water, if the waste is not handled responsibly.

Toluene was also used in the synthesis procedure, which can affect the brain and central nervous system with chronic exposure (Breysse, 2017).

It should also be noted that the synthesis of the C18 sorbent involves several similar reagents, such as AIBN activator, several monomers including stearyl methacrylate and poly (ethylene glycol) methyl ether methacrylate and a crosslinker, ethylene dimethacrylate. But it also contains completely different reagents compared to the MIP synthesis in this study, such as: fused silica and 3-(trimethoxysilyl)propyl methacrylate (a silanization reagent). Porogens are also employed in the C18 sorbent synthesis, such as 1-propanol and 1,4-butanediol (Li *et al.,* 2011). The activator, monomers and crosslinker are all bonded in the synthesis procedure and subsequently generate minimal waste. The fused silica does not have significant environmental impacts and the porogens have a similar impact compared to the MIP porogen.

The few millilitres of toluene used during the MIP synthesis seems insignificant when compared to the large volumes used in industry and the environmental impact of the pesticide template. In conclusion, synthesis of the MIPs has a potentially greater environmental impact than the commercial C18 and NIP sorbents because of the template used that needs to be washed out before the MIP is useable. This potential impact can of course be minimised if the generated waste is handled with care and treated correctly.

5.4: Cost comparison of the synthesised MIPs compared to the C18 sorbent

Table 5.1 summarises the cost calculation for 1 g MIP and NIP synthesis based on the molar ratios for the standard MIP as used in this study. Firstly, only the chemical cost was brought into consideration. The high purity pesticide templates were by far the most expensive component per 1 g MIP; acetochlor more so than the others.

Table 5.1: Cost calculation for the preparation of 1 g raw NIP and MIP

Synthesis components	Amount required	Cost per 1 g	Cost per required amount	Cost per 1 g final polymer
MAA	284.0 mg	R1.35	R0.38	**R41.03**
EDGMA	653.9 mg	R5.27	R3.45	
AIBN	3921.7 µL	R7.50	R29.41	
Toluene	1857.7 µL	R4.19	R7.78	
Templates				
Atrazine	8.9 mg	R1 785.0	R15.92	**R56.94**
Terbuthylazine	9.4 mg	R1 515.0	R14.26	**R55.29**

Synthesis components	Amount required	Cost per 1 g	Cost per required amount	Cost per 1 g final polymer
Acetochlor	11.1 mg	R9 960.0	R110.19	**R151.22**
Alachlor	11.1 mg	R1 975.0	R21.85	**R62.88**

Table 5.1 shows it costs R41.03 to prepare 1 g of NIP. The triazine MIPs are cheaper to prepare than the chloroacetamide MIPs. The bulk polymer C18-E sorbent can be purchased from Merck for R6934.00 for 100 g, thus at R69.34 per 1 g in bulk. This is comparable to the final prices calculated for each MIP per 1 g as in shown Table 5.1, except for acetochlor MIP, at almost double the price. From a cost perspective, the acetochlor MIP would thus probably not be useful for routine analysis purposes.

The empty SPE cartridges cost R1208.00 for a pack of 30, or R40.27 for an empty SPE cartridge. The average cost for 200 mg of polymer was calculated at R14.69. This calculates to R54.96 per self-manufactured MIP cartridge. The Phenomenex SPEs (supplied by Separations) were R 2078.30 for a pack of 30 with 500 mg C18-E sorbent each. This calculates to R 69.29 per cartridge. From the same supplier, a 50 pack with 100 mg C18-E sorbent costs R1439, thus R 28.78 per cartridge. This indicates the MISPE cartridges are not exceptionally more expensive to produce than purchasing the C18 SPE cartridges.

These calculations are an underestimate, as some MIP and NIP polymer is lost during size fractionation. There were also no labour costs or preparation consumables included such as vials and pipette tips, as well as waste disposal costs. The total cost can be estimated to be at least an additional R40.00 per cartridge, thus an estimated total of R94.96 per self-manufactured MISPE cartridge. The MTMIP would be substantially more expensive.

5.5: Future work

Inhibitor removal

It was deemed unnecessary to remove the 4-methoxyphenol polymerization inhibitor, firstly because the polymerization reaction occurred without its removal. Secondly, as size fractionation was performed to provide a selected size fraction of the synthesised polymers, strict control of the polymer reaction was not considered a necessity.

A specific bead size polymer can be prepared, with the focus on optimizing the packing integrity and ease. Most of the problems encountered during the study involved channelling in the packed SPE sorbent and a larger particle size may improve the integrity of the packed sorbent. A larger particle size sorbent will, however, adversely affect the adsorption capacity of the MISPE, as the surface to volume ratio of the polymer particles will decrease with larger particle sizes, thus less surface area will

be available for imprinting and also for exposure to the sample solution for the same volume of sorbent. Not grinding or crushing the particles after MIP synthesis will reduce waste and may result in more active pores on the surface as less of them may be damaged during the size fractionation process. Perhaps some compromise can be reached between particle size, the size fractionation performed and eventual adsorption capacity of the MISPE. The adsorption capacity in this study was found to be more than adequate for the maximum residue limits of the pesticides. Thus a decrease will not necessarily negatively affect the functionality of the MISPEs.

Dummy templates

A major concern for pesticide extraction and subsequent analysis utilising the MISPE is a false positive result due to pesticide, originally used as template, contaminating the sample. Measures can and were of course taken to prevent this, such as the optimization and validation of the template removal procedure. The MISPE was also washed or conditioned with methanol prior to sample loading to ensure any residual template pesticide was removed before the sample was introduced. The methanol fraction used for conditioning was also analysed utilising GC-MS to ensure no residual template was present in the eluate. Another solution is the use of a dummy template. The use of a dummy template MIP for the extraction of pesticides from cannabis has not been reported in the literature (in fact no literature could be found for MIPs used for the extraction of any cannabis or related products). It was demonstrated throughout this study that atrazine and terbuthylazine have affinity for MIPs prepared using either template, therefore both triazine pesticides can be extracted from the cannabis plant matrix with MISPE prepared using either of the triazine MIPs. The same was demonstrated for the chloroacetamide pesticides, in the sense that either chloroacetamide MIP could be effectively used for the extraction of both acetochlor and alachlor from the cannabis sample matrix. The use of less toxic and non-pesticide dummy templates will also reduce the environmental impact risk as discussed in Section 5.3, leading to less waste disposal concerns.

Solid phase extraction in different forms

There are many different shapes and sizes of solid phase extraction, such as dispersive solid phase extraction (dSPE) and solid phase microextraction (SPME). This study only focused on SPE with sorbent packed into a cartridge or syringe, but the use of MIPs in other forms in the extraction of target analytes might be beneficial or advantageous in some regards. SPME, for example, has less preparation steps and can be done much more quickly than conventional SPE, which might be beneficial for high throughput samples or environments. QuEChERS is a very popular technique for the extraction of pesticides from various matrices. Aspects of QuEChERS inspired the acetonitrile & salt extraction procedure for this study, however a MIP dSPE technique and other salts, such as $MgSO_4$,

have yet to be explored for their viability for the extraction of pesticides from the cannabis sample matrix.

The analysis of biologically accumulated pesticides

For this study only cannabis samples spiked with pesticides were analysed. Future studies should include a comparison in extraction behaviour and efficiencies of biologically accumulated pesticides in cannabis to spiked cannabis samples.

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Appendices

Department of Health- Licences to possess cannabis 2018……a

Department of Health- Licences to possess cannabis 2019……b

Department of Health- Licences to possess cannabis 2020……c

AccuStandard COA for atrazine 100 µg/mL- (P-008S) ……d

AccuStandard COA for alachlor 100 µg/mL- (P-102S) ……...r

AccuStandard COA for acetochlor 100 µg/mL- (P-465S) ……...f

AccuStandard COA for terbuthylazine 100 µg/mL- (P-465S) ……...g

PESTANAL™, analytical standard 99% atrazine (BCBR6277V) ……h

PESTANAL™, analytical standard 97.1% acetochlor (BCBV8448) ……i

PESTANAL™, analytical standard 99.3% alachlor (BCBW0992) ……j

PESTANAL™, analytical standard 98.3% terbuthylazine (BCBT2196) ……k

Additional fragmentation diagrams for terbuthylazine, acetochlor and alachlor ……l-p

Appendix a

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT, 1965 TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE PURPOSE OF EDUCATION.

Date of Issue: 09 February 2018	Expiry Date: 08 February 2019	Permit No: POS 020/2018/2019

Authority is hereby granted in terms of Section 22A (9)(a)(i) of the above-mentioned Act to **Mr P J Myburgh** of the **NAFS-109 Sovereign Dr, 21 Route 21 Business Park, Centurion, 0178** to acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **09 February 2018** to **08 February 2019**.

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year
Canabis Sativa	Schedule-7	200 g [two hundred grams]
Canabis Indica	Schedule-7	200 g [two hundred grams]

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **Analytical Research Purposes** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Mr P J Myburgh**

   ID Number: **920318 5039 089**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Mr P J Myburgh**

   ID Number: **920318 5039 089**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded **on this permit**.
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **NAFS-109 Sovereign Dr, 21 Route 21 Business Park, Centurion, 0178** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **08 February 2019** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

DIRECTOR-GENERAL:
DEPARTMENT OF HEALTH

DATE: 09 February 2018

Appendix b

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT 1965, (ACT 101 OF 1965) TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE PURPOSE OF EDUCATION

Date of Issue: 09 April 2019	Expiry Date: 08 April 2020	Permit No: POS 247/2019/2020

Authority is hereby granted in terms of Section 22A(9)(a)(i) of the above-mentioned Act to **Mr. P J Myburgh** of **National Analytical Forensic Services, 109 Sovereign Drive, R21 Corporate Park, Centurion, 0178** to acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **09 April 2019** to **08 April 2020**.

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year	
Cannabis Sativa	Schedule-7	200 g	[two hundred grams]
Cannabis Inidica	Schedule-7	200 g	[two hundred grams]

Total Items: **2**

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **Analytical Research Purpose** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Mr. P J Myburgh**

   ID Number: **920318 5039 089**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Mr. P J Myburgh**

   ID Number: **920318 5039 089**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded **on this permit**.
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **National Analytical Forensic Services, 109 Sovereign Drive, R21 Corporate Park, Centurion, 0178** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **08 April 2020** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

DIRECTOR-GENERAL:
DEPARTMENT OF HEALTH

DATE: 09 April 2019

Appendix c

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT 1965, (ACT 101 OF 1965) TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE ANALYTICAL RESEARCH PURPOSE

Date of Issue: 28 February 2020	Expiry Date: 27 February 2021	Permit No: POS 113/2020/2021

Authority is hereby granted in terms of Section 22A(9)(a)(i) of the above-mentioned Act to **Mr. Paul Josef Myburgh** of **National Analytical Forensic Services, 109 Sovereign Drive, R21 Corporate Park, Centurion, 0178** to acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **28 February 2020** to **27 February 2021.**

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year	
Cannabis Sativa	Schedule-7	200 g	[two hundred grams]
Cannabis Indica	Schedule-7	200 g	[two hundred grams]

Total Items: 2

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **Analytical Research Purpose** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Mr. Paul Josef Myburgh**

   ID Number: **920318 5039 089**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Mr. Paul Josef Myburgh**

   ID Number: **920318 5039 089**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded **on this permit**.
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **National Analytical Forensic Services, 109 Sovereign Drive, R21 Corporate Park, Centurion, 0178** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **27 February 2021** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

for K Ganasoodien (16/3)
DIRECTOR-GENERAL:
DEPARTMENT OF HEALTH

DATE: 28 February 2020

Appendix d

125 Market Street
New Haven, CT 06513
USA

AccuStandard,® Inc.

Tel (203)786-5290
Fax (203)786-5287
www.AccuStandard.com

CERTIFICATE OF ANALYSIS

Catalog No: P-005S
Description: Atrazine
Lot: 217031413
Solvent: Methanol
Hazards: *Refer to SDS for complete safety information*

Signal Word: Danger

Date Certified: Mar 29, 2017
Expiration: Apr 29, 2019
Sample Size: 1 mL
Components: 1
Storage Condition: Freeze (<-10 °C)

Included on ISO/IEC 17025 Scope of Accreditation: Yes

Included on ISO Guide 34 Scope of Accreditation: Yes

Component	CAS #	Purity % (GC/FID)	Prepared Concentration[1] (µg/mL)	Certified Analyte Concentration[2] (µg/mL)
Atrazine	1912-24-9	100.0	100.1	100.1

A product with a suffix (-1A, -2B, etc. or -01, -02, etc.) on its lot number has had its expiration date extended and is identical to the same lot number without the suffix.

[1] All weights are traceable through NIST, Test No. 822-275872-11

[2] Certified Analyte Concentration = Purity x Prepared Concentration.

The Uncertainty associated with the gravimetric values reported on this certificate is ±0.24%. This value is the expanded uncertainty and represents an estimated standard deviation equal to the positive square root of the total variation of the uncertainty of components. A normal distribution is assumed and a coverage factor of K=2 is chosen using approximately a 95% confidence level.

Labels and certificates follow U.S. Conventions in reporting numerical values: A comma (,) is used to seperate units of one-thousand or greater. A period (.) is used as a decimal place marker.

See reverse side for additional information

Certified By: Larry Decker, Organic QC Manager

For use in routine laboratory analysis.

Appendix e

125 Market Street
New Haven, CT 06513
USA

AccuStandard® Inc.

Tel (203)786-5290
Fax (203)786-5287
www.AccuStandard.com

CERTIFICATE OF ANALYSIS

Catalog No: P-102S
Description: Alachlor
Lot: 216071296
Solvent: Methanol
Hazards: **HIGHLY FLAMMABLE** - *Refer to SDS for safety info*

Danger 2

Date Certified: Jul 25, 2016
Expiration: Jul 25, 2020
Sample Size: 1 mL
Components: 1
Storage Condition: Ambient (>5 °C)

Included on ISO/IEC 17025 Scope of Accreditation: Yes

Included on ISO Guide 34 Scope of Accreditation: Yes

Component	CAS #	Purity % (GC/FID)	Prepared Concentration[1] (µg/mL)	Certified Analyte Concentration[2] (µg/mL)
Alachlor	15972-60-8	99.4	100.2	99.6

A product with a suffix (-1A, -2B, etc. or -01, -02, etc.) on its lot number has had its expiration date extended and is identical to the same lot number without the suffix.

[1] All weights are traceable through NIST, Test No. 822-275872-11

[2] Certified Analyte Concentration = Purity x Prepared Concentration. The Uncertainty associated with the gravimetric values reported on this certificate is ±0.24%. The CRM Uncertainty calculated for this product is ±5%. These values are the expanded uncertainty and represent an estimated standard deviation equal to the positive square root of the total variation of the uncertainty of components. A normal distribution is assumed and a coverage factor of K=2 is chosen using approximately a 95% confidence level.

Labels and certificates follow U.S. Conventions in reporting numerical values: A comma (,) is used to separate units of one-thousand or greater. A period (.) is used as a decimal place marker.

See reverse side for additional information

Certified By: Larry Decker, Organic QC Manager

For use in routine laboratory analysis.

Appendix f

125 Market Street
New Haven, CT 06513
USA

AccuStandard® Inc.

Tel (203)786-5290
Fax (203)786-5287
www.AccuStandard.com

CERTIFICATE OF ANALYSIS

Catalog No: P-465S
Description: Acetochlor
Lot: 217031406
Solvent: Methanol
Hazards: *Refer to SDS for complete safety information*

Signal Word: Danger

Date Certified: Mar 29, 2017
Expiration: Mar 29, 2020
Sample Size: 1 mL
Components: 1
Storage Condition: Ambient (>5 °C)

Included on ISO/IEC 17025 Scope of Accreditation: Yes

Included on ISO Guide 34 Scope of Accreditation: Yes

Component	CAS #	Purity % (GC/MS)	Prepared Concentration[1] (µg/mL)	Certified Analyte Concentration[2] (µg/mL)
Acetochlor	34256-82-1	99.0	100.9	99.9

A product with a suffix (-1A, -2B, etc. or -01, -02, etc.) on its lot number has had its expiration date extended and is identical to the same lot number without the suffix.

[1] All weights are traceable through NIST, Test No. 822-275872-11

[2] Certified Analyte Concentration = Purity x Prepared Concentration.

The Uncertainty associated with the gravimetric values reported on this certificate is ±0.24%. This value is the expanded uncertainty and represents an estimated standard deviation equal to the positive square root of the total variation of the uncertainty of components. A normal distribution is assumed and a coverage factor of K=2 is chosen using approximately a 95% confidence level.

Labels and certificates follow U.S. Conventions in reporting numerical values: A comma (,) is used to separate units of one-thousand or greater. A period (.) is used as a decimal place marker.

See reverse side for additional information

Certified By:

Larry Decker, Organic QC Manager

For use in routine laboratory analysis.

Appendix g

125 Market Street
New Haven, CT 06513
USA

AccuStandard® Inc.

Tel (203)786-5290
Fax (203)786-5287
www.AccuStandard.com

CERTIFICATE OF ANALYSIS

Catalog No: P-169S
Description: Terbuthylazine Standard
Lot: 216111229
Solvent: Methanol
Hazards: *Refer to SDS for complete safety information*

Signal Word: Danger

Date Certified: Nov 17, 2016
Expiration: Nov 17, 2019
Sample Size: 1 mL
Components: 1
Storage Condition: Ambient (>5 °C)

Included on ISO/IEC 17025 Scope of Accreditation: Yes

Included on ISO Guide 34 Scope of Accreditation: Yes

Component	CAS #	Purity % (GC/MS)	Prepared Concentration[1] (µg/mL)	Certified Analyte Concentration[2] (µg/mL)
Terbuthylazine	5915-41-3	100.0	100.3	100.3

A product with a suffix (-1A, -2B, etc. or -01, -02, etc.) on its lot number has had its expiration date extended and is identical to the same lot number without the suffix.

[1] All weights are traceable through NIST, Test No. 822-275872-11

[2] Certified Analyte Concentration = Purity x Prepared Concentration.

The Uncertainty associated with the gravimetric values reported on this certificate is ±0.24%. This value is the expanded uncertainty and represents an estimated standard deviation equal to the positive square root of the total variation of the uncertainty of components. A normal distribution is assumed and a coverage factor of K=2 is chosen using approximately a 95% confidence level.

Labels and certificates follow U.S. Conventions in reporting numerical values. A comma (,) is used to separate units of one-thousand or greater. A period (.) is used as a decimal place marker.

See reverse side for additional information

Certified By: Larry Decker, Organic QC Manager

For use in routine laboratory analysis.

SIGMA-ALDRICH®
3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	ATRAZINE PESTANAL™, analytical standard
Product Number:	45330
Batch Number:	BCBR6277V
Brand:	Sigma
CAS Number:	1912-24-9
Formula:	$C_8H_{14}ClN_5$
Formula Weight:	215.68
Expiration Date:	20 JAN 2021
Quality Release Date:	18 FEB 2016

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	WHITE TO OFF WHITE	WHITE
APPEARANCE (FORM)	POWDER OR CRYSTALS	POWDER
PURITY (HPLC AREA %)	≥ 98.0 %	99.0 %
MELTING POINT	173 - 180 C	177 C
WATER	≤ 1.0 %	0.05 %
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

SIGMA-ALDRICH

3050 Spruce Street, Saint Louis, MO 63103 USA
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Certificate of Analysis

Product Name:	ACETOCHLOR PESTANAL™, analytical standard
Product Number:	33379
Batch Number:	BCBV8448
Brand:	Sigma-Aldrich
CAS Number:	34256-82-1
Formula:	$C_{14}H_{20}ClNO_2$
Formula Weight:	269.77
Expiration Date:	AUG 2022
Quality Release Date:	14 SEP 2017

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	COLORLESS TO YELLOW AND COLORLESS TO LIGHT BROWN--YELLOW	LIGHT BROWN-YELLOW
APPEARANCE (FORM)	LIQUID	LIQUID
PURITY (GC AREA %)	≥ 95.0 %	97.1 %
REFRACTIVE INDEX N20/D	1.5248 - 1.5288	1.5268
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

SIGMA-ALDRICH®

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Certificate of Analysis

Product Name:	ALACHLOR PESTANAL™, analytical standard
Product Number:	45316
Batch Number:	BCBW0992
Brand:	Sigma-Aldrich
CAS Number:	15972-60-8
Formula:	$C_{14}H_{20}ClNO_2$
Formula Weight:	269.77
Expiration Date:	OCT 2022
Quality Release Date:	08 NOV 2017

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	WHITE TO OFF WHITE	WHITE
APPEARANCE (FORM)	POWDER OR CRYSTALS	CRYSTALS
PURITY (GC AREA %)	≥ 98.0 %	99.3 %
MELTING POINT	40 - 45 C	42 C
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

Dr. Claudia Geltner
Manager Quality Control
Buchs, Switzerland

SIGMA-ALDRICH

3050 Spruce Street, Saint Louis, MO 63103 USA
Email USA: techserv@sial.com Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:	TERBUTHYLAZINE PESTANAL™, analytical standard
Product Number:	45678
Batch Number:	BCBT2196
Brand:	Sigma
CAS Number:	5915-41-3
Formula:	$C_9H_{16}ClN_5$
Formula Weight:	229.71
Expiration Date:	OCT 2021
Quality Release Date:	11 NOV 2016

TEST	SPECIFICATION	RESULT
APPEARANCE (COLOR)	WHITE TO LIGHT YELLOW AND FAINT BEIGE TO LIGHT BEIGE	OFF WHITE
APPEARANCE (FORM)	POWDER OR CRYSTALS	POWDER
PURITY (GC AREA %)	≥ 98.0 %	98.3 %
MELTING POINT	174 - 179 C	175 C
WATER	≤ 1.0 %	0.10 %
PROTON NMR SPECTRUM	CONFORMS TO STRUCTURE	CONFORMS

Dr. Claudia Geitner
Manager Quality Control
Buchs, Switzerland

Additional fragmentation diagrams and mass spectra for terbuthylazine, acetochlor and alachlor

Terbuthylazine has the following molecular formula: $C_9H_{16}ClN_5$ and thus also has two theoretical molecular ions at an *m/z* of 229 (76%) and 231 (24%) due to the Cl isotope abundance. Figure A.1 shows the EI mass spectrum obtained from the NIST library. The three largest mass fragments are 214; 173 and 43. Figure A.2 shows the mass fragments on the chemical structure for terbuthylazine. Their chemical formulas were calculated as follows:

- *m/z* 229 and 231 relates to M+
- *m/z* 214 and 216 relates to [M-15] due to loss of CH_3 to $C_8H_{13}ClN_5^+$
- *m/z* 173 and 175 relates to [M-56] loss of C_4H_8 to $C_5H_8ClN_5^+$
- *m/z* 43 relates to [M-186] $CH_3CH_2N^+$

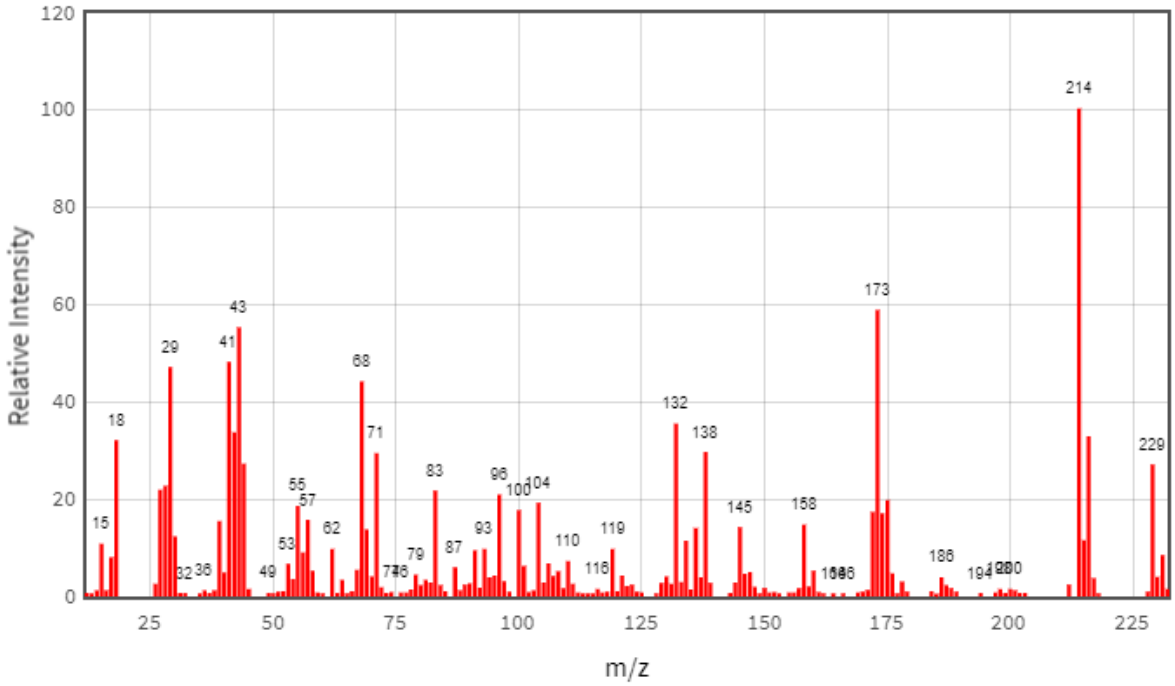

Figure A.1: Electron ionization mass spectrum for terbuthylazine from the NIST library depicting the relative intensities of the expected fragmentation pattern (NIST, 2014)

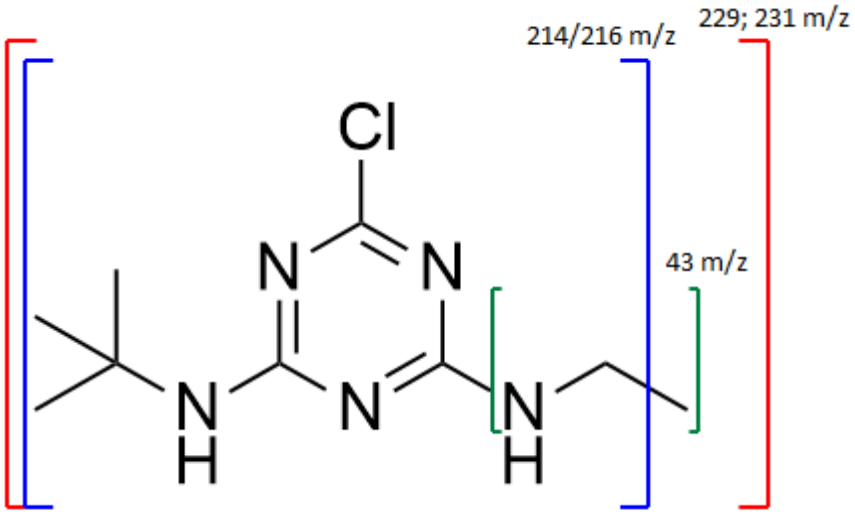

Figure A.2: Terbuthylazine mass fragmentation diagram depicting where the major fragments might be formed on the representation of the chemical structure

The fragments of *m/z* 173 and 175 are quite prominent in both the atrazine and terbuthylazine EI mass spectra and a possible solution for the chemical structure is shown in Figure A.3

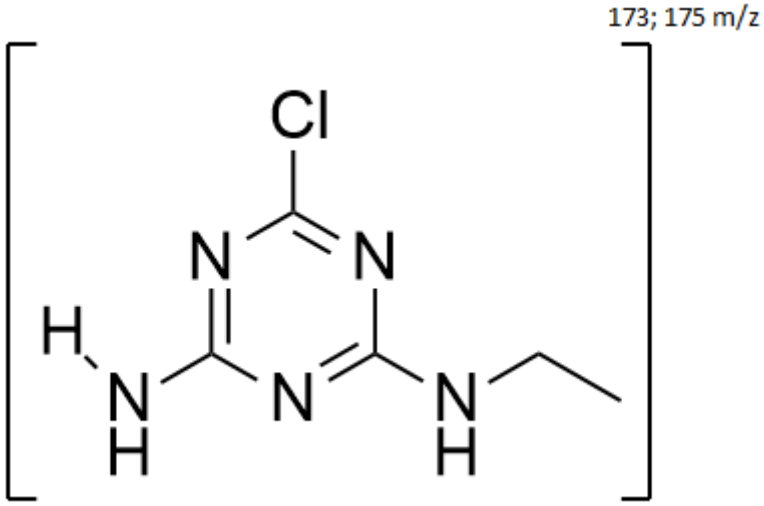

Figure A.3: A plausible m/z 173 and 175 mass fragment for both atrazine and terbuthylazine

Acetochlor has the mass spectra as seen in Figure A.4 and the following molecular formula: $C_{14}H_{20}ClNO_2$ with a molecular mass of 269 (76%) and 271 (24%) due to the Cl isotope abundance. The following chemical formulas were calculated for prominent *m/z* fragments of acetochlor:

- *m/z* 269 and 271 relates to M+ with molecular formula of $C_{14}H_{20}ClNO_2^+$
- *m/z* 223 and 225 relates to [M-46] due to loss of C_2H_6O to $C_{12}H_{14}ClNO^+$
- *m/z* 146 relates to [M-123] loss of $C_4H_8ClO_2$ to $C_{10}H_{12}N^+$
- *m/z* 59 relates to [M-210] $CH_3CH_2OCH_2^+$

Figure A.5 and A.6 shows possible solutions to the chemical structures of the fragments.

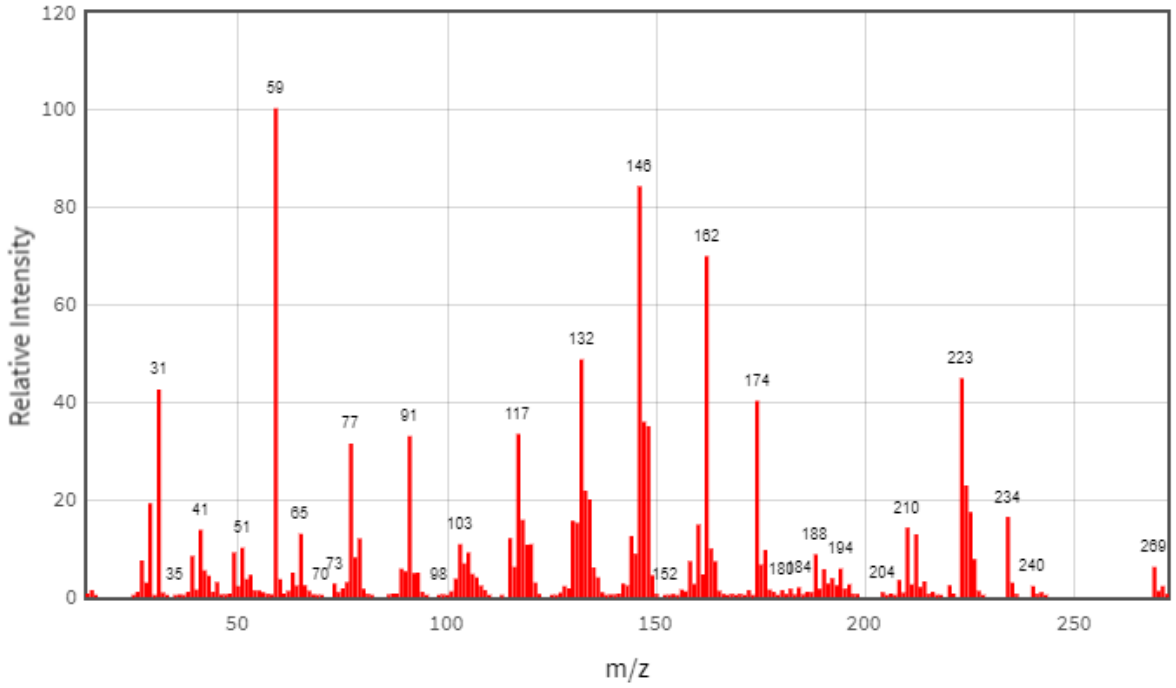

Figure A.4: Acetochlor is known as 2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)-acetamide in the NIST library. This is the EI mass spectrum for acetochlor with the relative intensities of the expected fragmentation pattern (NIST, 2014)

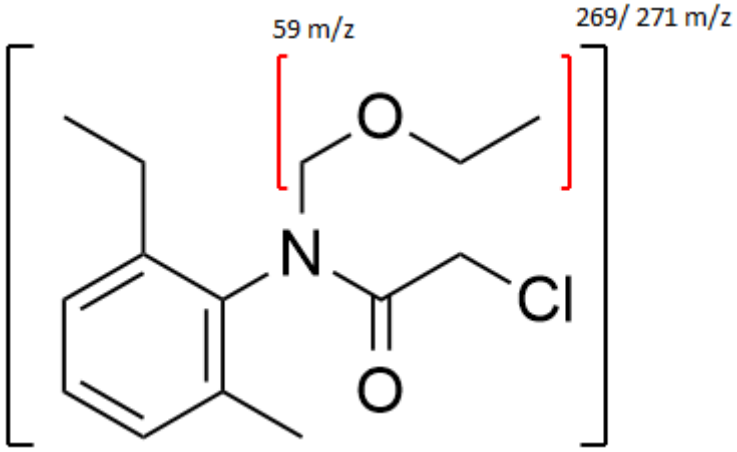

Figure A.5: Acetochlor molecular mass and the most abundant m/z 59 fragment. This mass fragment does not have the expected Cl isotope ratio abundance in the mass spectrum, thus further confirming the fragmentation pattern as indicated

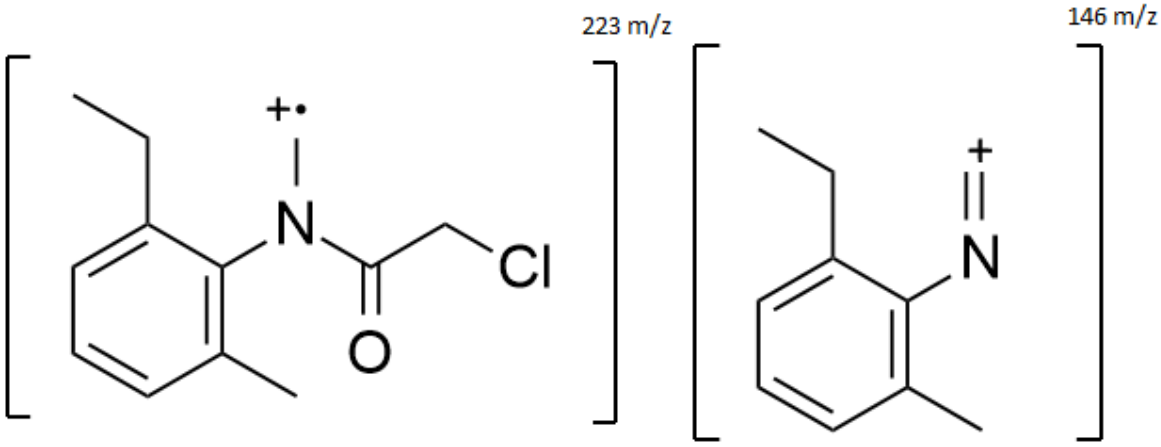

Figure A.6: Plausible structures for the large relative intensity mass fragments with an m/z of 223 and 146 observed in the EI mass spectrum of acetochlor

Alachlor has the mass spectra as seen in Figure A.7 and the following molecular formula: $C_{14}H_{20}ClNO_2$ with a molecular mass of *m/z* 269 Da (76%) and 271 Da (24%) due to the Cl isotope abundance. The following are calculations for possible chemical formulas for prominent fragments observed in the mass spectrum:

- *m/z* 269 and 271 relates to M+ with molecular formula of $C_{14}H_{20}ClNO_2^+$
- *m/z* 188 relates to [M-81] due to loss of $C_1H_2ClO_2$ to $C_{13}H_{18}N^+$
- *m/z* 160 relates to [M-109] due to loss of $C_3H_6ClO_2$ to $C_{11}H_{14}N^+$
- *m/z* 45 relates to [M-210] $CH_3OCH_2^+$

Figure A.7 and A.8 shows possible solutions for the chemical structures for the above fragments

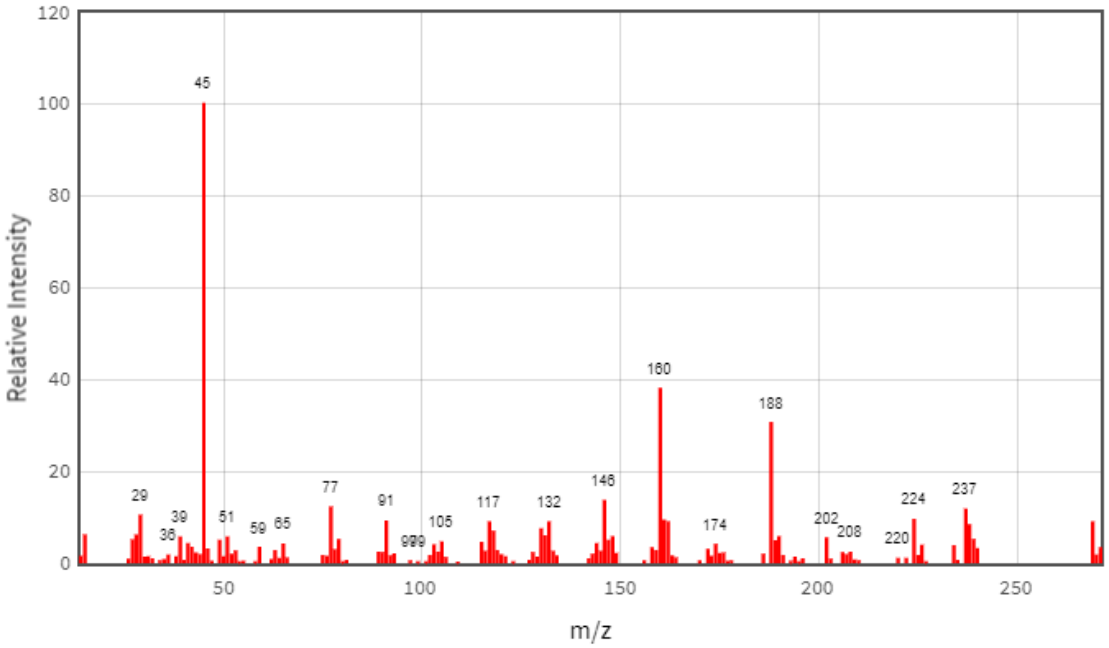

Figure A.7: Electron ionization mass spectrum for alachlor from the NIST library with the relative intensities for the expected fragmentation pattern (NIST, 2014)

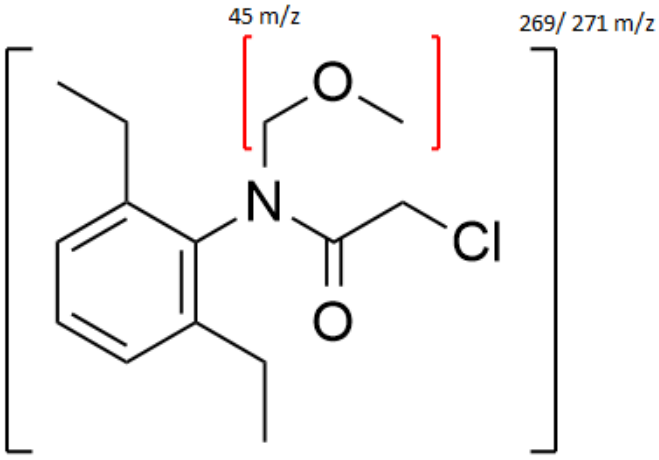

Figure A.8: The molecular mass fragment and the high relative intensity m/z 45 mass fragment observed in the alachlor mass spectrum

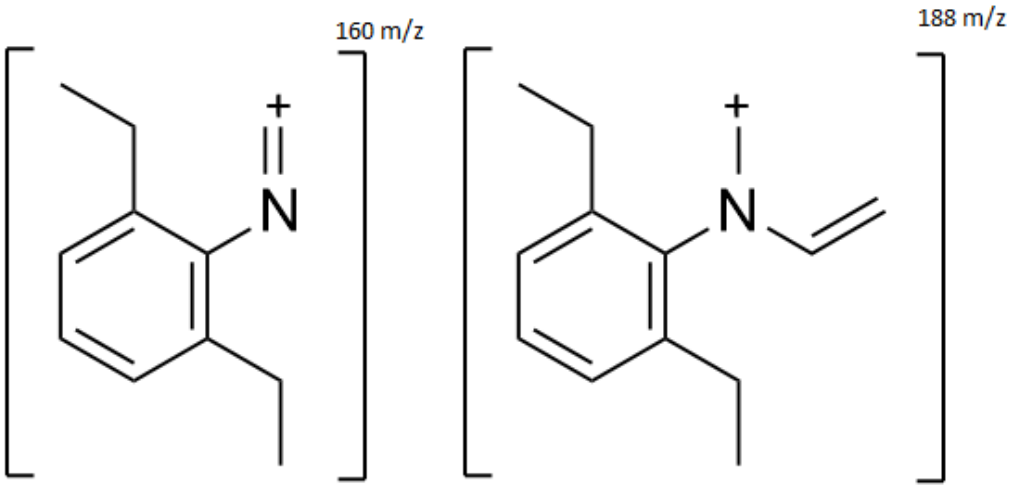

Figure A.9: The molecular mass fragments plausibly responsible for the observed m/z 160 and 188 in the EI mass spectrum of alachlor