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METHOD DEVELOPMENT BASED ON MOLECULARLY IMPRINTED POLYMERS FOR THE SELECTIVE EXTRACTION OF ORGANIC COMPOUNDS IN COMPLEX AQUEOUS MATRICES

By

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science

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WITS University, Johannesburg, 2009

DECLARATION

I declare that this research report is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

(Signature of Candidate)

----------Day of----------2009

ABSTRACT

Contamination of water from organic compounds in low concentrations such as triazines herbicides and estrogens and the fact that due to complex matrix effect, the compounds are not easy to determine which call for sample preparation approaches such as MIPSE – MMLLE. A selective extraction method based on the combination of microporous membrane liquid-liquid extraction (MMLLE) and molecularly imprinted polymers (MIPs) was developed and applied to the extraction of 17 β-estradiol and selected triazines herbicides in complex aqueous samples. These pollutants were extracted from aqueous complex samples through the hydrophobic porous membrane that was impregnated with toluene, which also formed part of the acceptor phase. In the acceptor phase, the compounds were re-extracted onto MIP particles. The extraction technique was optimised for the amount of MIP particles in the organic acceptor phase, extraction time, and type of organic acceptor solvent and desorption solvent. Triazines herbicides (simazine, atrazine and propazine) and estrogenic compound (17 β-estradiol) were used as model compounds in this study. HPLC-UV was used for the determination of these organic compounds.

An extraction time of 90 minutes and 10 mg of MIP were found to be optimum parameters for triazines extraction. Toluene as the acceptor phase was found to give higher triazines binding onto MIP particles compared to hexane and combinations of diethyl ether and hexane. 90% methanol in water was found to be the best desorption solvent compared to acetonitrile, methanol and water. Higher MIP mass (30 mg) was used for 17 β-estradiol (E2) because of poor binding kinetics. An extraction time of 60 minutes and 90% methanol in water were adopted. Hexane: ethyl acetate (3:2) as the acceptor phase was found to give a stable membrane and give better partitioning of the E2 from the aqueous donor. In order to improve the binding of the E2 onto the MIP ultrasound assisted rebinding was studied.

The developed method proved its selectivity to the extraction of complex matrices where clean chromatograms were obtained. Furthermore samples spiked with varying amounts of humic substances were also extracted and “clean” chromatograms obtained. Reference

materials containing ametryn, prometon, prometryn, propazine, simetryn, simazine and terbutryn were extracted to demonstrate the validity of the developed technique. The results indicated that the MMLLE – MIP could be used to reliably determine real samples. The method was then applied to spiked lettuce, apple extracts and wastewater where clean chromatograms were obtained compared to MMLLE alone or to the microporous membrane liquid-liquid extraction – non imprinted polymer combination (MMLLE – NIP).

The triazine MIP showed a certain degree of group specificity and the extraction efficiency was 68 % for simazine, 74 % for atrazine and 69% for propazine, giving enrichment factors of 40, 56 and 45, respectively in deionised water. The extraction efficiency in apple extracts were 55%, 63% and 60% for simazine, atrazine and propazine respectively. The percentage relative standard deviation in plant samples ranged from 1 to 15. The E2 MIP also demonstrated high selectivity although the extraction efficiencies and enrichments factors were low due to poor E2 binding on the MIP particles.

“To my wife and kids, I love you”.

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Firstly I would like to thank the LORD my savior for the intelligence and wisdom. Special thanks to **Prof Luke Chimuka** for his, mentorship and support that he gave me throughout my stay at The University of the Witwatersrand. Without his understanding and financial support, I could not have made it this far. Many thanks also go to **Prof Ewa Cukrowska** for her significance academic and resources support that she gave me through my studies. Equipment, chemicals, computers and research material were never an issue because of your support. To Dr. Hlanganani Tutu I would like to say your inputs, seminars and guidance were very valuable.

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I would also like to dedicate this work to my son **Ashley**, my daughter **Lenny**, my wife **Nonhlanhla** and the rest of my family members not forgetting my parents. I love you all. To my Pastor, **Innocent Sibanda** and his wife **Litracy**, your wisdom and spiritual guidance was and remains important in my life.

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LIST OF ABREVIATIONS

% RSD	Percentage relative standard deviation
ACCN	1,1 Azobis(cyclohexane carbonitrile)
AIBN	2,2' –Azobis(2-isobutyronitrile)
C_{18}	Octadecyl
CRM	Certified reference material
E1	Estrone
E2	17 β-estradiol
E3	Estriol
E_A,	Extraction efficiency
ECD	Electron capture detector
EDCs	Endocrine disrupting compounds
EDMA	Ethylene glycol dimethacrylate
EE2	17 α ethynylestradiol
ELISA	Enzyme-linked immunosorbent assay
En	Enrichment factor
EPA	Environmental Protection Agency
FID	Flame ionisation detector
GC	Gas chromatograph
HF-MMLLE	Hollow-fibre microporous membrane liquid-liquid extraction
HPLC	High performance liquid chromatograph
HSs	Humic substances
IC	Ion chromatograph
IS	Immunosorbents
ISSPE	Immunosorbents solid phase extraction
LLE	Liquid liquid extraction
LM	Liquid membrane
LME	Liquid membrane extraction
MAA	Methacrylic acid
MASE	Membrane assisted solvent extraction

MeEE2	Mestranol
MI	Molecular imprinting
MIPs	Molecularly imprinted polymers
MISPE	Molecularly imprinted solid phase extraction
MMLLE	Microporous membrane liquid liquid extraction
MMPE	Multimodal and mixed mode phase extraction
NIP	Non imprinted polymer
PDMS	Polydimethylsiloxane
PTFE	Polytetrafluroethylene
RAM	Restricted access membrane
SBSE	Stir bar sorptive extraction
SIA	Sequentia injection analysis
SLM	Supported liquid membrane extraction
SPE	Solid phase extraction
SPMD	Semi-permeable membrane devices
SPME	Solid phase microextraction
TOPO	Trioctyl phosphine oxide
TRIM	Trimethylopropane
UV	Ultra violet

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 INTRODUCTION

Point and non - point source contamination of water resources by organic chemicals such as triazine herbicides and estrogens has been a major water quality issue. Triazine herbicides are commonly used as selective pre and post - emergency herbicides for the control of broadleaf and grassy weeds in many agricultural crops. They act by binding to specific proteins in the thylakoid membranes of chloroplast, where they inhibit the Hill reaction (photolysis of water) and hence block photosynthesis. Their prolonged use involves the risk of their retention on crops and soil from which in turn, due to washing and leaching processes, these substances pass to surface and groundwater. Therefore it is necessary to monitor the levels of triazines herbicides and their fate in the environment.

Estrogens such as 17 β-estradiol (E2) and triazines belong to a class of chemicals called endocrine disrupting compounds (EDCs). These are defined as exogenous compounds that alter the function of the endocrine systems and consequently cause adverse health effects in an intact organism, or its progeny. EDCs are of global concern due to their widespread occurrence, persistence, bioaccumulation and possible adverse effects on natural ecosystem and human health. These chemicals may originate from natural processes or agricultural processes (Liu *et al.,* 2004).

In recent years, there has been increasing attention towards the potential effects of EDCs in aquatic environments on human and wildlife endocrine systems eg ferminisation of male fish, abnormal reproductive processes and the development of testicular and prostate cancer even at the low concentration down to 1ng l^{-1} in humans (Sumpter *et al.,* 1995; Crain *et al.,* 1998; Desbrow *et al.,* 1998; Routeledge *et al.,* 1998). EDCs associated to these effects include natural compounds such as estrogens, progesterons and phytoestrogens, synthetic compounds such as 17 α ethynylestradiol and a range of organic pollutants, including pesticides, surfactants and plasticizers (Campbell *et al.,* 2006; Richardson, 2002; Orton *et al.,* 2009; www.epa.gov (accessed April 22, 2009)).

According to an European Union Directive (EEC Drinking water Directive, 1998), the maximum concentration limit for atrazine is set at 0.1 μg l^{-1} for a single substance and 0.5 μg l^{-1} for sum of all pesticides in waters samples. The alarm threshold value is set at 3 μg l^{-1} in surface water (Brouwer *et al.,* 1995). In food samples the limits are in the range of between 100 μg kg^{-1} and 250 μg kg^{-1} according to the Environmental Protection Agency (EPA) Tolerance Index (www.epa.gov, accessed April 22, 2009)).

In most instances the triazine herbicides and 17 β-estradiol are found in complex water samples such as wastewater, biological fluids and fruit samples. Quantitative and qualitative analysis of these compounds from these complex matrices is faced with so many interferences from the sample matrix hence sample preparation and clean up steps are of paramount importance prior to analysis.

The most preferably used sample clean-up technique is solid phase extraction (SPE) (Sabik, 2000). However, when more complex samples are cleaned up, some difficulties are observed (Hennion, 1999). Often the matrix components are difficult to remove and this may lead to coextraction of other matrix components which may later interfere with the signals originated from the target analyte (Khrolenko, 2002). Generally the inconvenience is reduced by dilution, but it can lead to the increase of the measurement errors such as compound not being detected (Khrolenko, 2002). The selective solid phase extraction sorbents that are suitable for complex samples are immunosorbents (IS), molecularly imprinted polymers (MIPs) and multimodal and mixed phase extraction (MMPE) (Ferrer and Barceló, 1999).

One limitation of MIPs prepared by noncovalent approach is that the interactions with the target analyte are mostly due to hydrogen bonding (Thordason *et al.*, 2000). This means that when the extraction is to be perfomed in aqeous solutions, water molecules will compete with the target analyte molecules in terms of hydrogen bonding and this leads to the loss of selectivity of the MIP. Yoshizako *et al.*, (1998) suggested that to eliminate such competition, the extraction of the target analyte using the MIP should be perfomed in an organic solvent media. This brings in further challenges since many pollutants are in aqueous media.

Combining macroporous membrane liquid - liquid extraction (MMLLE) and MIP extraction may offer a possibility to avoid the limitations associated with hydrogen binding. MMLLE technique is a partially selective simple technique which involves the extraction of an analyte into an organic phase through a microporous membrane

filled with the organic liquid. It is believed that if the MIP is incoporated into the acceptor phase of the MMLLE the limitations associated with hydrogen binding may be solved and much cleaner extracts may be obtained. Thus the combination of two techniques in one step has the potential to provide unsurpassed selectivity.

1.2 BACKGROUND OF THE STUDY

Liquid liquid extraction (LLE) is the oldest sample preparation technique. However, LLE technique is now less popular because of its drawbacks of being time consuming, not easy to automate and consuming large organic solvents. Other alternative sample preparation techniques for aqueous samples are solid phase extraction (SPE) (Hennion, 1993), solid phase microextraction (SPME) (Beltran *et al.,* 2000), stir bar sorptive extraction (David *et al.,* 2007) and liquid membranes (Johnsson and Mathiasson, 2007).

Pinto *et al., (*2000) reported the use of an octadecyl, C_{18} bonded silica for preconcentration prior to HPLC for the determination of triazines in water. Martinez *et al.,* (2000) reported the use of C_{18} bonded silica prior to the determination using capillary electrophoresis. Ferrer *et al.,* (2002) and Chapius *et al.,* (2005) reported the preconcentration of triazine herbicides from aqueous samples by direct percolation through the MIPs. This present problems since water molecules interfere with hydrogen bonding between MIP binding sites and the analytes (Dauwe *et al.,* 1996). Pap *et al.,* (2002) reported that a small amount of water affects the selectivity of the MIP hence a drying step was included in the procedure. Other reseachers (e.g.

Chapius *et al.,* 2003 and Baggiani *et al.,* 2001) have attempted to use the MIP sorbent for selective elution instead of extraction.

One approach for the extraction of complex samples is to combine two different sample preparation techniques. Such combination has the potential to produce synergism with high selectivity. Thordason *et al., (*2000) reported to have combined supported liquid membrane extraction technique with immnunoassays for the separation and determination of phenols in water samples so as to increase the selectivity. Supported liquid membrane (SLM) extraction prior to SPE has also been investigated for atrazine enrichment and clean up from complex matrices such as fruit juices extracts (Khrolenko *et al.,* 2000). In comparison to SPE from juice samples, the application of SLM – SPE enrichment provided much cleaner extracts that lowered the detection limit. Other researchers (Haginaka *et al.,* 2007 and Koeber *et al.,* 2001) have combined restricted access media (RAM) with molecularly imprinted polymers. One such study was performed by Haginaka *et al.,* (2007) on rat plasma where a RAM polymer was imprinted to produce a RAM- MIP material with enhanced selectivity. Bjarnasson *et al.,* (1999) used a MIP column coupled to a SPE (C_{18}) one in the extraction of triazines from complex samples such as river water, urine and fruit extracts. This combination resulted in very high selectivity especially in water with high humic acids.

In this project, an attempt is made to combine macroporous membrane liquid - liquid extraction (MMLLE) and MIPs. Selectivity in MMLLE is based mainly on the differences in partition coefficients between the analyte and potential intereferring

matrices into the organic liquid used as the acceptor. On the hand, MIP is much more selective since analyte extraction is based on the size, shape and structure.

It is believed that if the MIP is incoporated in the acceptor phase of the MMLLE technique, the problems mentioned above may be solved and much cleaner extracts may be obtained. To demonstrate the potential of MMLLE-MIP technique, triazine herbicides (simazine, atrazine and propazine) and 17 β-estradiol were chosen as model compounds.

1.3 STATEMENT OF THE PROBLEM

Analysis of chemicals in complex samples such as those of plant and biological origin is still a challenge. Using selective extraction sorbents such as immunosorbents (IS) or molecularly imprinted polymers (MIPs) alone may not always give clean extracts. The new approach is to combine two or more sample extraction techniques.

1.4 PURPOSE OF THE STUDY

The purpose of the project was to develop a techinique for the selective extraction and preconcentration of organic analytes from complex aqueous samples such as wastewater and plant extract. The method is based on the combination of the macroporous membrane liquid - liquild extraction (MMLLE) and the molecularly imprinted polymer (MIP) techniques. The MIP is incoporated into the organic phase of the MMLLE to avoid direct aqueous extraction onto to the MIP. The selectivity

and extraction efficiency was determined and compared to those obtained by MMLLE only.

1.5 THE OBJECTIVES OF THE PROJECT

The objectives of the study are:

i. To prepare the polymers (MIPs) in the laboratory for atrazine and 17 β-estradiol.
ii. To develop a method which enables the selective extraction and preconcentration of organic compounds based on the combination of MMLLE and the MIP.
iii. To optimise the developed method to achieve better selectivity and extraction efficiency.
iv. To compare the selectivity of the proposed technique to that of the MMLLE only.
v. To apply the developed and optimised method to the extraction of real complex aqeous samples such as wastewater and plant extracts.

1.6 HYPOTHESIS

The combination of MIPs and MMLLE can solve some of the problems faced by these individual techniques in extraction and pre-concentration of organic compounds from complex aqueous samples especially enhancing selectivity.

1.7 IMPORTANCE OF THE PROJECT

Reliable environmental analysis of chemicals such as triazine and E2 is a prerequisite for their risk assessment. Pesticides residue analysis plays an important role in food quality for evaluating food safety and possible risk to human health. The analytical methods used for the determination of organic compounds in complex aqueous samples may require sample extraction, clean up and pre-concentration steps because the compounds normally exist in low concentrations.

The traditional sample preparation techniques such as liquid-liquid extraction is now less popular because of problems such as time consuming, not easy to automate and consuming large organic solvents. Other sample preparation techniques such as SPE lack selectivity and may not be good for complex samples. MIPs that are selective can be employed in the pre-concentration of chemicals from aqueous samples. One limitation of MIPs especialy prepared by noncovalent approach is that the interactions with the target analyte are mostly due to hydrogen bonding. This means that when the extraction is perfomed in aqeous solutions, water molecules will compete with the target analyte molecules in terms of hydrogen bonding and and this leads to the loss of selectivity of the MIP. Therefore to eliminate such competition, the extraction of the target analyte using the MIP should be perfomed in an organic solvent media. This brings in further challenges since many pollutants are in aqueous media.

It is believed that if the MIP is incoporated in the acceptor phase of the MMLLE technique, the limitations associated with hydrogen binding may be solved and much

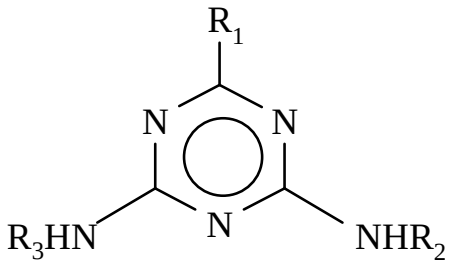
cleaner extracts may be obtained. The MMLLE-MIP has the potential to produce clean and preconcentrated extracts.

CHAPTER 2: LITERATURE REVIEW

2.1 THE OCCURRENCE AND FATE OF TRIAZINE HERBICIDES

2.1.1 The occurrence of triazine herbicides

Triazine herbicides have been banned in many countries. However because of their early widespread usage and persistence, are still of environmental concern. Numerous studies have been conducted to investigate the occurrence, fate and the effects on human health and the environment from the extensive use of herbicides to control weeds (Koplin *et al.,* 1998 and Stamer *et al.,* 1998a). Triazines (Figure 2.1) and their degradation products as an example were found in shallow aquifers across the Midwestern United States in early 1990s (see Table 2.1 below) (Stamer *et al.,* 1998a).



Compound	R_1	R_2	R_3
Simazine	Cl	CH_2CH_3	CH_2CH_3
Atrazine	Cl	$CH(CH_3)_2$	CH_2CH_3
Propazine	Cl	$CH(CH_{3)2}$	$CH(CH_3)_2$

Figure 2.1: The structures of triazine compounds

Table 2.1: Triazine herbicides analysed in samples collected from 76 Midwestern reservoir outflows from April 1992 through September 1993 (Kolpin *et al.,* 1996).

Herbicide	**Detection limit (µg l^{-1})**	**Mean concentrations (µg l^{-1}) in detectable samples**
Atrazine	0.05	1.36
Propazine	0.05	0.10
Simazine	0.05	0.21

In the UK, atrazine was detected in 5 out of 11 estuaries with a maximum concentration of 0.38 µg l^{-1} whilst simazine was detected in 6 of the estuaries with a maximum concentration of 0.39 µg l^{-1} (SAC Scientific, 1987). It should be mentioned that deethylatrazine was detected more frequently than diisopropylatrazine and that supports conclusion from field-dissipation studies during the 1990s that deethylation is the more stable, biotic degradation pathway (Adams and Thurman, 1991; Kolpin *et al.,* 1994)

2.1.2 The fate of triazine herbicides in the environment

Physiochemical properties shown in Table 2.2 below indicate that soluble and mobile herbicides, such as atrazine, propazine and simazine are transported primarily in the dissolved phase hence their aquatic fate is strongly influenced by their moderate solubilities and their persistence.

Table 2.2: The physicochemical properties of the triazine herbicides (Wauchope *et al.,* 1992)

Herbicide	**Water solubility ($mg\ l^{-1}$)**	**$LogP_{ow}$**	**Soil sorption coefficient (K_{oc} $ml\ g^{-1}$)**	**pKa**	**Vapor Pressure (mPa)**
Atrazine	33	2.2	100	1.7	0.040
Propazine	5	2.5	154	1.7	0.004
Simazine	6.2	3.0	138	1.6	0.001

The compounds are not volatile and losses to the atmosphere is therefore likely to be minimal. Herbicides with K_{oc} values less than about 500 ml g^{-1} tend to be transported primarily in the dissolved phase where as K_{oc} values greater than 1000 ml g^{-1} are transported primarily on suspended-sediments particles (Becker *et al.,* 1989; Goolsby *et al.,* 1992).

Once herbicides are in surface water, they degrade much slower than in soil because surface water contains much less organic matter and fewer micro-organisms to degrade the herbicides (Scribner *et al.,* 2005). The main routes of removal of herbicides from water are photo-enhanced hydrolysis to 2-hydroxy derivatives, adsorption onto sediments and degradation by micro-organisms. However adsorption is believed to be rapid and reversible.

Atrazine and propazine degrade slowly to deethylatriazine by deethylation. Simazine and atrazine degrade to deisopropylatrazine by dealkylation. The degradation products are more soluble and mobile than the parent compound. The removal of an ethyl side-chain from atrazine relative to the removal of an isopropyl side-chain is preferred (Scribner *et al.,* 2005).

2.2 OCCURRENCES AND FATE OF 17 β - ESTRADIOL

2.2.1 The occurrence of 17 β-estradiol (E2).

The natural hormones such as E2 shown in Figure 2.1 are excreted by human beings and female animals. E2 is secreted by the adrenal cortex, testes, ovary and placenta in human and animals. The estrogenic compounds end up in the environment through sewage discharge and animal waste disposal. The steroid hormones have been detected in effluents of the sewage treatment plants (STPs) and surface water (Desbrow *et al.,* 1998, Kuch and Ballschitter, 2001, and Terms *et al.,* 1999).

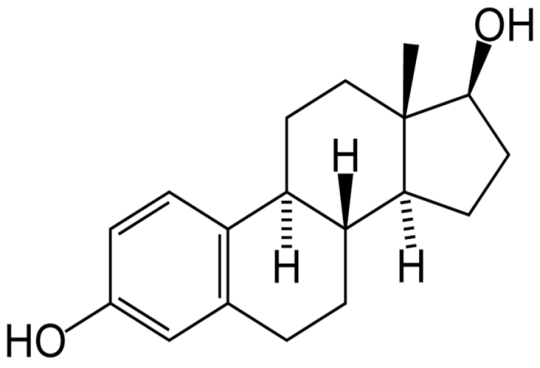


Figure 2.2: The structure of 17 β-estradiol (E2)

Average concentration of E2 in influents of six Italian activated sludge STPs was 12 ng l^{-1} (Baronti *et al.,* 2000). In Brazilian STPs E2 was detected with an average concentration of 21 ng l^{-1} (Ternes *et al.,* 1999a). The concentrations of E2 in influents of Japanese STPs ranged from 30 – 901 ng l^{-1} in autumn and 20 – 941 ng l^{-1} in summer (Nasu *et al.,* 2000).

2.2.2 The fate of E2

Sewage discharge containing E2 end up in aquatic system. The physicochemical properties of the estrogens (Table 2.3 below), shows that they are hydrophobic compounds hence they may adhere to hydrophobic sediments and part of it may undergo degradation although the bulk of the compounds remain in aqueous solution.

Table 2.3: Physicochemical properties of Steroids (EDC) (Lai *et al.,* 2000).

Chemical Name	**Molecular weight (g)**	**Water solubility (mg l^{-1}) @ 20°C)**	**Vapour pressure (mm Hg)**	**Log K_{ow}**
Estrone	270.4	13	2.3×10^{-10}	3.43
17B-estradiol (E2)	272.4	13	2.3×10^{-10}	3.94
Estriol (E3)	288.4	13	2.3×10^{-15}	2.81
17 α ethynylestradiol (EE2)	296.4	4.8	2.3×10^{-11}	4.15
Mestranol (MeEE2)	310.4	0.3	2.3×10^{-10}	4.67

E2 with a sorption constant K_{oc} of 3300 and logK_{ow} of 3.94 (Lai *et al.,* 2000, William *et al.,* 1999) is hydrophobic in nature and has high binding with sediment/soil

particles. The sorption of estrogens to the sediments correlates to the presence of organic carbon content and increases with the salinity in water (Lai *et al.,* 2000). Peterson *et al.,* (2000) and Shore *et al.,* (1995a) reportedly to have found E2 in ground water. Shore *et al.,* (1995a) believed that a constant E2 concentration of about 5 ng l^{-1} in spring waters was caused by infiltration of E2 through the soil profile to the ground water. Although the K_{oc} and the K_{ow} values of E2 suggest hydrophobicity and high binding to the sediments/soil particles, E2 has been detected not only in surface water but also in groundwater. This clearly shows that there is need to better understand their behavior in different environment media (Ying *et al.*, 2002). The fate of estrogens once bound to sediments is an important consideration in terms of transportation and potential exposure to organisms.

2.2.3 Degradation of E2

In humans and animals, E2 is rapidly oxidised to estrone (E1), which can be further converted to estriol (E3), the major excretion product. Many other polar metabolites like 16 - hydroxyestrone, 16 ketoestrone or 16 epiestriol are formed and can be present in urine and feaces. Many municipal wastewater treatment plants can reduce estrogenic compounds to some extent. Removal of estrogenic compounds is due to processes such as biodegradation and adsorption on sludge. In an aerobic batch experiments with activated sludge, E2 is oxidised to E1.

2.3 COMMON SAMPLE PREPARATION TECHNIQUES

2.3.1 Liquid – liquid extraction

Liquid liquid extraction (LLE) is the traditional technique for the extraction of organic analytes from aqeous solutions. The separation of compounds is based on their solution preferences for two different immiscible liquids, usually water and an organic solvent. It involves shaking a mixture of an aqeuous solution and an organic solvent in a separating funnel. Figure 2.2 below shows the apparatus used in LLE.

For the last decade, conventional LLE applications have dropped dramatically following the obligatory reduction of chlorinated solvent usage (Hennion, 1999).

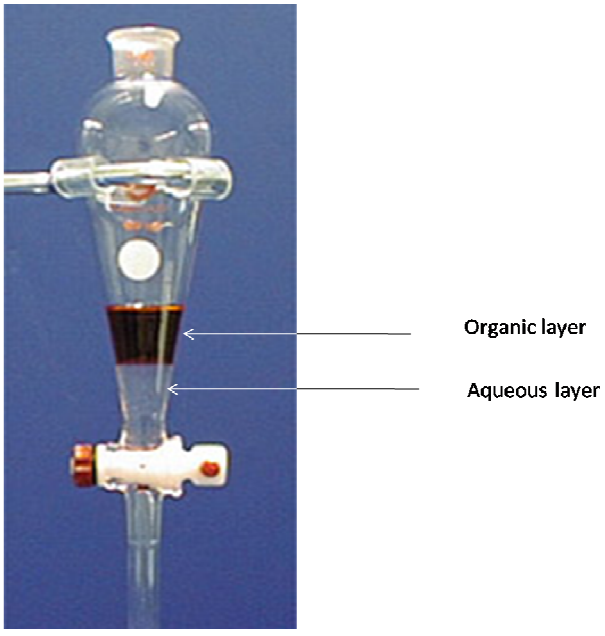


Figure 2.3: Apparatus used in conventional liquid -liquid extraction.

Besides the huge consumption of organic solvents, LLE has the disadvantages of requiring lengthy analysis times, automatiuon challenges and more clean up stages. In parallel, intensive research in the area of solid phase extraction has promoted the development of new formats and new sorbents (Rolcik *et al.,* 2002), thereby making SPE a method of choice rather than LLE. The advantages and disadvantages of LLE are summarised below.

Advantages

- It is simple and easy to perfom.
- Large enrichment factors can be obtained.
- Large range of organic solvents to choose from.

Disadvantages

- Uses large organic solvents often not environmental friendly.
- Labour and time intensive as when extracting complex matrixes which demands many steps including organic solvent reduction and clean up.
- Formation of emulsions which often make it difficult to separate the phases.
- Lack of selectivity for complex samples such as waste water and biological fluids.
- Not easy to automate.

Examples of the use of LLE in extraction of organic compounds

Sabik, (1994) extracted pesticides from waters samples and sediments. In this study, five hundred nanograms of each compound were added to 50 ml of a water sample. The extraction was carried out with three successive portions of 20 ml of organic solvent. The following solvents were tested: ethyl acetate, dichloromethane, ethyl acetate-isopropanol (50:50, v/v) and ethyl acetate-hexane (80:20 and 90:10, v/v). The results showed that none of them gave satisfactory results (per cent recovery < 30%). Shiavon, (1988) reported the extraction of triazines and its degradation products from soils, followed by concentration of the aqueous extract through lyophilisation.

2.3.2 Liquid membrane extraction (LME)

The use of membrane based extractions are increasingly seen as offering an alternative to conventional sample preparation in analysis of chemical species in both food and agricultural samples (Chimuka *et al.,* 2006). LME extraction techniques differ from the LLE in that a membrane impregnated with a liquid is put between the donor and acceptor phases and therefore does not allow the mixing of the two solutions. The organic compounds partition from the donor phase into the acceptor phase through a membrane. The driving force being the difference in concentration gradient. Changing conditions in acceptor phase such as making the target analytes ionize increases the diffusion gradient and hence the selectivity. Membrane extraction can provide high concentration enrichments factors as well high selectivity.

The selectivity of the membrane is based on the pore size and the pore size distribution. Bigger molecules are excluded from passing into the acceptor phase

from the donor phase. Polar compounds are also excluded on the basis that they can not disssolve in the organic solvent or hydrophobic membranes. There are two main types of liquid membrane extraction techniques depending on whether acceptor phase is aqueous, supported liquid membrane (SLM) or organic liquid, microporous membrane liquid liquid extraction (MMLLE) (Figure 2.3).

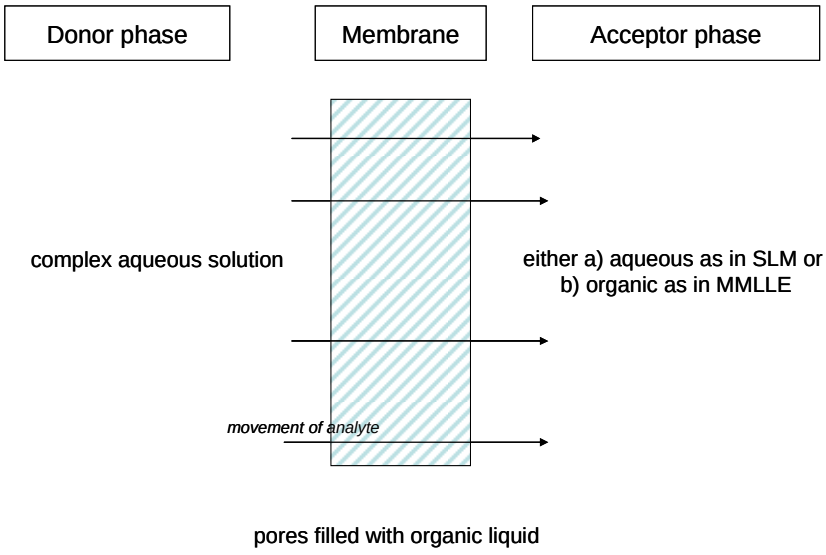


Figure 2.4. Schematic diagram showing the two types of LME process.

The advantages of liquid membrane extraction are summarised below:

The LME utilises a small amount of solvent and can still provide an excellent clean up and extraction efficiency depending on the sample matrix and liquid membrane technique. On line connection can be acheived i.e can be connected to say a GC for direct determination after extraction especially with MMLLE and to LC after extraction with SLM. With LME, the conditions in the donor, acceptor phases and

membrane can be modified and high selectivity and enrichment factors can be achieved. Automation can be easily achieved.

Disadvantages of liquid membrane extraction are as follows:

For complex samples such as plant extracts and sediments, selectivity obtained from LME may not be good enough, though this depends on the membrane technique. In LME, memory effect can be a problem especially for hydrophobic compounds. This is common in membranes with bigger thickness. Leakage of the donor phase onto the acceptor phase leading to membrane instability especially in MMLLE can also be a problem. This also depends on the configuration of the membrane technique.

Supported liquid membrane (SLM) technique

This is a three-phase extraction technique. Analytes are extracted from an aqueous donor phase into another aqueous acceptor phase through an organic liquid. The organic phase is held between the aqueous phases by a porous, inert hydrophobic supporting membrane. Capillary forces in the pores hold the organic liquid. Thus, two different equilibria are involved, which makes the system chemically analogous to extraction and back-extraction in classic liquid–liquid extraction. The analytes are partitioned from the aqeous sample into the organic membrane and are then re-extracted into the aqeous acceptor phase. The concentration gradient across the two aqueuos phases is the driving force. In order to maintain the concentration gradient across the two phases, the target solutes must be able to exist in two different forms, e.g in an nonionic form on the donor side and ionised form in the acceptor side in

order to be irrevessibly trapped. This can be achived by adjusting the pH in the two aqueous phases (Chimuka *et al.,* 2004). The back-extraction step considerably increases the selectivity of the extraction. Also, supported liquid membrane extraction provides unique possibilities for extracting polar, ionisable and even permanently charged compounds, including metal ions, which are more difficult to extract with other techniques. This can be achieved by incoporatiing a carrier to transport the solutes across the membrane.

Supported liquid membrane extraction is applicable to analytes of high or moderate polarity with Log K_{ow} (octanol-water partition coefficient) in the range of 1.5 to 3.0 although Thordarsson *et al.,* (2000) and Tudorache *et al.,* (2004) reported the extraction of non polar compounds, 4-nitrophenol and atrazine with modification.

Megersa *et al.,* (1999) developed a method for the preconcentration of methoxy-s-triazine herbicides based on the SLM extraction technique. The analytes were extracted from a donor solution of pH 7 into a porous PTFE membrane impregnated with di-n-hexyl ether and trapped in the acidic acceptor of pH 1. Cleaner chromatograms were obtained as compared to SPE and sub ppb concentrations were extracted. SLM extraction has been also successfully applied to determination and quantification of triazine herbicides in aqueous matrices. Chimuka *et al.,* (1997) described the application of supported liquid membrane extraction for the analysis of triazine in natural waters. SLM has been applied to environmental and biological samples as seen in review articles (Cordero *et al.,* 2000, Jonsson *et al.,* 1999 and Chimuka *et al.,* 2002). Jung *et al.,* (2002) extracted phenolic compounds in a

circulating nutrient solution containing a lot of humic acids using modified SLM extraction procedure (Figure 2.4). Diexylether containing 5% trioctylphosphine oxide (TOPO) was used as membrane liquid and 0.1 M Na_3PO_4 was used in the acceptor phase. TOPO was added into the membrane to increase the partitioning thus increasing the overall mass transfer and extraction efficiency.

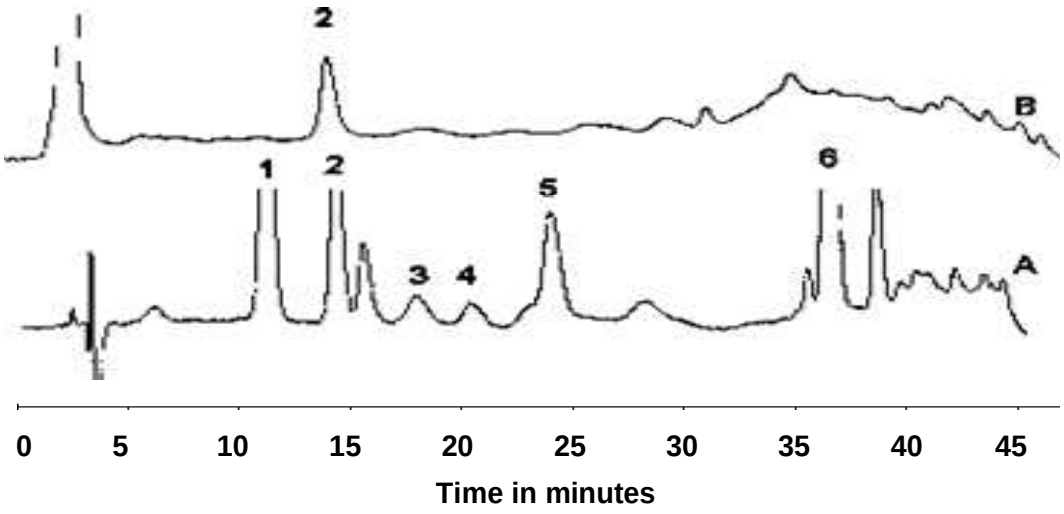


Figure 2.5: Standards mixture of compounds (A) and corresponding circulating nutrient sample solution after SLM extraction (B). (1) p-hydroxybenzoic acid, (2) vanillic acid, (3) salicylic acid, (4) benzoic acid, (5) ferulic acid, and (6) phenazine-1-carboxylic acid (Jung *et al.*, (2002).

Liquid membrane instability often cited as a major limitation of SLM extraction technique is caused by decline of analyte flux or even leakage of one aqeous phase into the other due to solvent or carrier loss during extraction. Factors such as

difference in osmotic pressure between the phases and emulsion formation have been identified as the cause of instability.

Microporous membrane liquid–liquid extraction (MMLLE)

This type of liquid membrane extraction is based upon a two-phase system, with one aqueous phase and one organic phase. Thus, it is equivalent to a single-step liquid–liquid extraction. A microporous hydrophobic membrane separates the two phases, and the organic phase also fills the pores of the membrane to provide a direct contact through a liquid–liquid interface without mixing the phases (Jönsson *et al.,* 2001, Jönsson 2002, Shen *et al.,* 1998). The analytes partition from the aqueous into the organic in the pores and difuse into the bulk organic due to difference in the concentration gradient.

The membrane used to separate the aqueous and organic phases can be a thin, inert polymeric membrane made from silicone rubber or polytetrafluoroethylene (PTFE). The concentration enrichment factor is therefore determined by the partition coefficient of the pollutant into the organic liquid. Jönsson *et al.,* (1999) reported that the highest enrichment factor that can be obtained is equal to the partition coefficient of the pollutant into the organic liquid.

One way to improve the mass transfer and hence the extraction efficiency is to maintain a big concentration gradient between the two phases. This can be achieved by continuosly pumping the the acceptor phase removing the target molecules from the acceptor. This however dilutes the extracted analytes.

In MMLLE, polar and charged compounds are poorly extracted as they poorly partition into the organic liquid. Macromolecules are excluded as they can not pass through the small pores of the membrane. Therefore only small uncharged organic pollutants can be selectively extracted into the acceptor phase. Addition of a carrier that is soluble in the organic liquid can enhance the extraction of more polar organic compounds and permanently charged molecules (Chimuka *et al.,* 2004).

Jonsson and Mathiasson (1999; 2002) reported the use of MMLLE to environmental and biological samples. Martinez *et al.,* (1995; 1996) reported a membrane based extraction procedure using hexane as the acceptor for the determination of triazines from sunflower and corn oils, and water samples respectively. Zorita *et al.,* (2008) utilised a hollow fibre filled with the organic liquid in a hollow-fibre microporous membrane liquid-liquid extraction (HF-MMLLE) in the extraction of steroids homornes from sewage samples. Detection limits in the low ng l^{-1} range were achieved when a GC-MS was used in the final analysis.

Hyötyläinen and coworkers (2002), were able to determine 18 pesticides in wine by the online coupling of a membrane extractor to GC system. Toluene was used as the extraction solvent (acceptor). Although the sample extraction took 40 minutes, the extract was clean with very few peaks from the wine matrix observed (Figure 2.5). Enrichment factors were as high as 17 with the average being around 7.5.

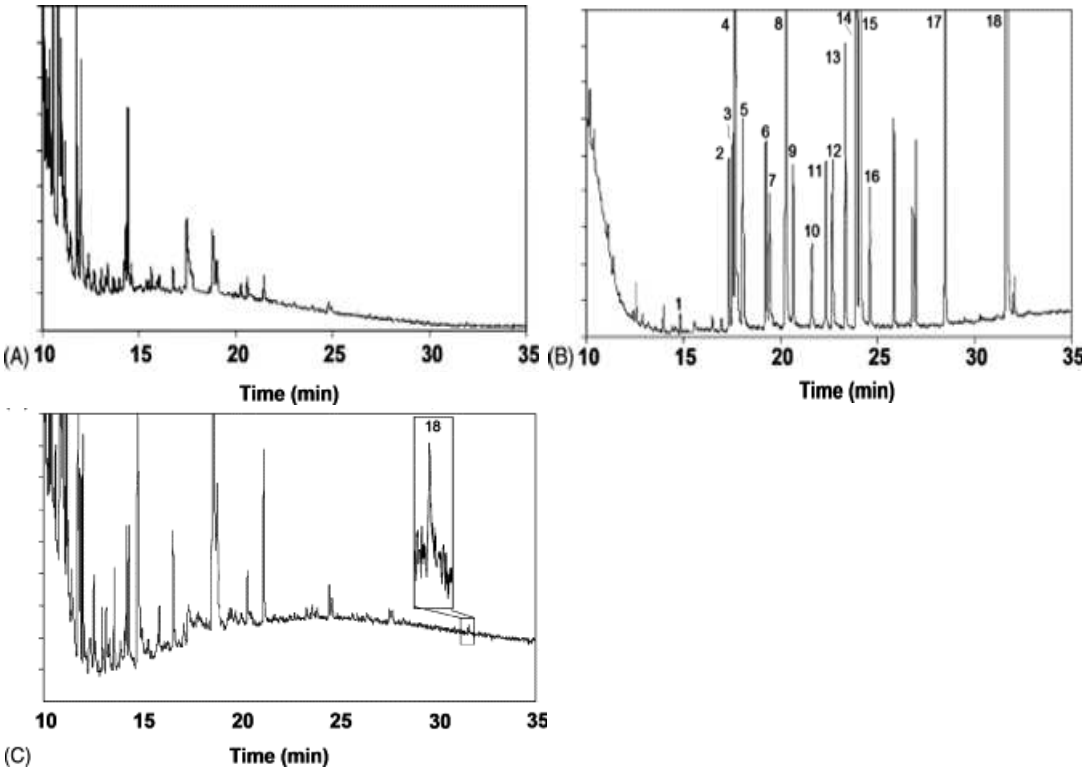


Figure 2.6: MMLLE-GC-FID determination of (A) a blank wine, (B) a MMLLE extract of a spiked red wine sample (c = 0.05 mg l^{-1}) and (C) a MMLLE extract of an Italian red wine containing tetradifon. Peak identification: 1, aldicarb; 2, diphenylamine (ISTD); 3, simazine; 4, atrazine; 5, lindane; 6, terbuthylazine; 7, metoxuron; 8, metobromuron; 9, vinclozolin; 10, isoproturon; 11, chlortoluron; 12, metazachlor; 13, quinalphos; 14, procymidone; 15, endosulfan 1; 16, endosulfan II; 17, endosulfan sulfate; 18, tetradifon (Hyötyläinen *et al.*, 2002).

2.3.3 Solid phase extraction

Solid phase extraction (SPE) is the mostly widely used technique for the preparation and preconcentration of samples. It is prefered to the classical liquid-liquid extraction

technique. SPE involves a partitioning between the liquid and the solid where the solid material is the extracting/sorbent material. SPE has been widely used to preconcentrate and remove pollutants from environmental and food samples. The sorbents typically used in SPE are silica based reversed phase (C_8, C_{18}), normal phase, ion exchange (cation and anion exchange) and adsorption based phases (alumina, graphitised carbon, silica gel).

Although solid phase extraction technique is the widely prefered sample preparation techinique over classical liquid-liquid extraction, its lack of selectivity is the major drawback. For example when a C_{18} sorbent is used, all non polar analytes compete for retention. This drawback has lead to the development of selective sorbents that will be discussed in later sections.

Most solid phase extraction techniques follows four basic steps (preconditioning, sample loading, washing and elution). Figure 2.6 below shows the steps involved in solid phase extraction. Preconditioning step is the activation of the sorbent surfaces by passing solvents like methanol and acetonitrile. Preconditioning is then followed by loading of the sample. The third step involves washing the catridge with a solvent that will not disrupt the bonding between the target analytes and the sorbent. The last stage, elution step is then achieved by passing a more stronger solvent with more affinity for the target analyte.

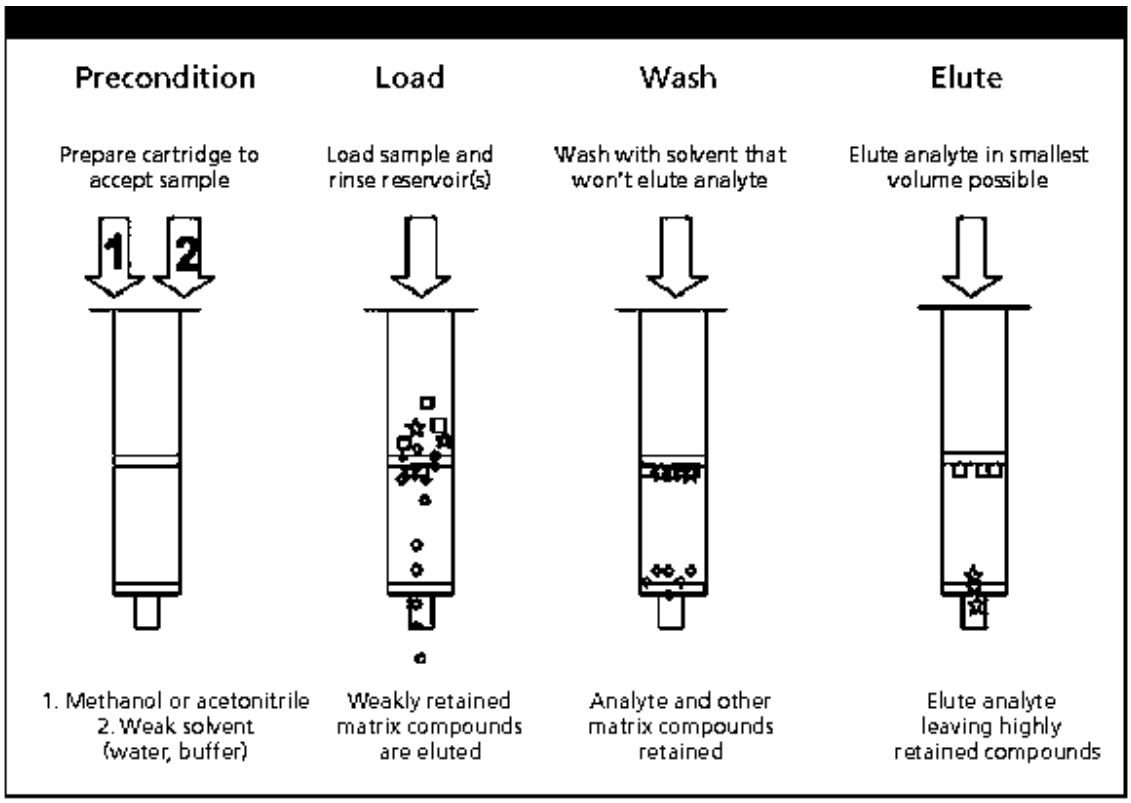


Figure 2.7: Schematic diagram representing the steps involved in solid phase extraction

SPE uses much less solvent therefore meaning that less amount of solvent will be disposed. Depending on the stationary phase SPE usually provides cleaner extracts and more reliable results. Many samples can be analysed in a short space of time. With SPE, automation can be easily achieved and be coupled to HPLC.

The most common disadvantage of the solid phase extraction is that it is not very selective in extracting a particular analyte except as ion exchange and other modern sorbents such as molecularly imprinted polymers (MIPs) and immunosorbents (IS).

Common non-selective solid phase extraction sorbents

There is a wide range of SPE sorbents that are used. Some of the sorbents are non-selective and the emerging ones are selective. In this section the common non selective sorbents are discussed.

Reversed phase (bonded silica)

This is the most popular SPE sorbent used in sample preparation of organic analytes. The most popular bonded silica is octadecyl (C_{18}). C_8 has been used in some cases. Reverse-phase SPE are employed to extract non-polar and moderately polar compounds such as pesticides, herbicides, hydrocarbons, steroids, fat soluble vitamins etc from polar samples such as water. Retention of an analyte is primarily due to nonpolar-nonpolar interactions and van der Waals or dispersion forces. The retention of compounds on the bonded silica correlates to the octanol-water partition coefficient ($\log K_{ow}$). Organic compounds with $\log K_{ow}$ above 3 normally give 95% recovery.

To obtain a better extraction efficiency and a totally apolar material, the trends are to minimise the number of residual silanol groups of the original silica. For this purpose, a trifunctional silane is used for bonding the n-alkyl chains and endcapping is carried out with trimethylsilane after bonding (Hennion, 1999).

Pinto and Jardim (2000) reported the use of SPE (C_{18}), in the extraction of triazines residues in water and subsequent detemination by a high-perfomance liquid

chromatography. Clean chromatograms were obtained but the extractions were not performed from a complex matrix samples but on tap and irrigation water. Khrolenko *et al.,* (2002) employed a C_{18} catridge in the extraction and prencentration of triazine herbicides. The results shows that a single application of SPE for sample pretreatment is insufficient as a method for clean-up of juice samples with capillary electrophoresis for the separation and UV detection of triazines.

Normal phase (silica, cyano, amino).

Normal phase sorbents include bare silica, alumina and silica chemically modified by polar groups such as amino, cyano or diol groups. Analytes dissolved in samples made of a usually non polar organic solvent are handled. This can be achieved by evaporating an aqeous solution to dryness and subsequently dissolution with a non polar organic solvent such as hexane. Elution of the retained analytes is achieved by a solvent of increasing polarity.

Van der Hoff *et al.,* (1991) reported the clean up of water samples extracts for the determination of organochlorine and pyrethroid insecticides using silica cartridges. The extracts were obtained from water by LLE with hexane as organic solvents. Clean up was achieved with a cartridge packed with 100 mg of silica. Gustavson *et al.,* (2000) used a normal phase based dual-zone restricted-access sorbent for the removal of methyl oleate from a semipermemeable membrane device extract.

Ion exchange

Ionic or ionizable analytes can be extracted by ion-pair and ion-exchange sorbents. Polymer based ion exchange are better than silica based because they are applicable in a wide range of pH (Hennion, 1999). Ion exchange include cationic and anionic exchange. Cation exchangers include weak carboxylic acid and strong aromatic or non-aromatic sulphonic acid groups. These are meant to trap positively charged analytes. Weak anion-exchange groups are made of primary or secondary amino groups whereas strong anion exchangers include quaternary amine forms. These are meant to trap negatively charged chemicals.

The retention and elution of the analytes is pH depended. Retention occurs when the analytes are in an ionisable pH and elution is achieved when the analytes are in their neutral form otherwise elution can be achieved by using a solution with a stronger ionic strength.

Cation exchangers have been used for the on-line determination of aniline derivatives after chemical sample pretreatment based on the precipitation of calcium with oxalic acid and complexation of iron with EDTA (Nielen *et al.,* 1985). The preatreatment was done so as to eliminate the overloading of the exchanger with inorganic ions. Hennion and co-workers, (1993; 1995) reported the use of cation exchange SPE based on sulphonic acid for the preconcentation of a polar and water soluble aminotriazole pesticides which was not retained on a C_{18} silica or polymers particles.

Adsorption (Alumina, graphitized carbon and silica gel)

Separation in this case is due to interactions of compounds with unmodified materials. Separation of analytes may be due to hydrophobic and hydrophilic interactions depending on which solid phase is used. Porous graphite carbon (PGC) is a crystalline structure made of large graphite sheets held together by weak Van der Waals forces. It offers retention by both hydrophobic and electrostatic interactions. Alumina based packings have been used for anion exchange and adsoption extraction of polar compounds such as vitamins. Alumina based packings has wide range of interaction depending on the pH (Supelco guide to SPE, 1998).

2.3.4 Solid phase micro – extraction (SPME)

Solid - phase microextraction (SPME) was introduced by Pawliszyn and co-workers (1990). The trends in SPME were reviewed by Elsert and Pawliszyn, (1997). The SPME technique integrates extraction, concentration, and sample introduction into a single step. It is quick, highly sensitive and readily adapted to automation. SPME is an equilibrium extraction technique and not an exhaustive extraction. The maximum sensitivity is obtained at the equilibrium point. However it is not necessary to reach this point and extractions can instead be performed for a defined period of time.

SPME is essentially a two step process, firstly a fibre is immersed in the sample and target analytes partition between the aqueous matrix and the fibre coating, when equilibrium is reached the fibre is removed. The the fibre is exposed to the hot injection port of a gas chromatograph (GC) where the analytes are desorbed from the fibre and passed through the instrument in the usual manner. The fibre can also be

desorbed into liquid chromatograph eluent using a static or dynamic mode and several commercial interfaces are available.

SPME has found favour when compared to solvent extraction because of the reduction in organic solvent use. It can also provide high sensitivity and can be used for polar and non-polar analytes. Figure 2.7 below shows the components of SPME.

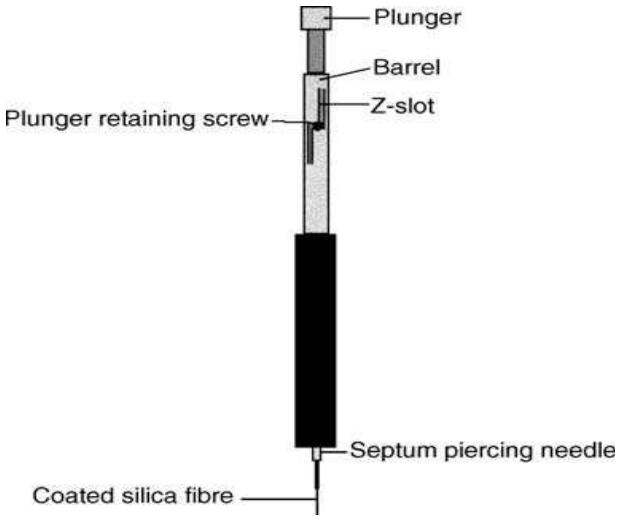


Figure 2.8: Components of SPME

Kin *et al.,* (2006) reportedly developed a method for the analysis of pesticides residues in vegetables and fruits samples using a GC-ECD after headspace solid phase micro-extraction. Limit of detection was between 0.001 to 0.01 mg kg^{-1}. SPME normally suffer from inteferences from the sample matrix but in this case matrix interferences were reduced by sample dilution. Elizabeth *et al.,* (2006), developed and optimised a method using SPME for the detection of seven organophosphate pesticides in drinking water treatment facility. Automated SPME

has been tested for steroid hormones analysis in environmental aqueous samples (Yang *et al.,* 2006).

Despite the citated advantages of SPME, it also has disavantages. Ridgeway *et al.,* (2007) stated that the limited volume of stationary phase that can be bound to the fibre lead to incomplete extraction and limit the sample enrichment capabilities. Besides its simplicity, SPME lacks selectivity when applied to complex matrices such as biological fluids, biological samples and wastewater samples. Matric effects can be an issue and quantitation generally requires matrix matched standards or the method of standard additions is needed. However recent publications have been reported where fibre containing MIP have been used (Djozan and Ebrahim, 2008: Prasad *et al.,* 2008). This offers better selectivity.

2.3.5 Stir bar sorptive extraction (SBSE)

Stir bar sorptive extraction (SBSE) was developed by Baltussen et al, (1999), to overcome the limited extraction capacity of SPME fibres. In SBSE, a large surface area offers high recoveries and extraction capacity. This is achieved by a glass stirrer bar coated with a thick bonded adsorbent layer (polydimethylsiloxane – PMDS) to give a large surface layer. As with SPME, thermal elution into a GC or solvent elution into a LC are also used for desoption of the bar.

The major advantage of SBSE technique is the high enrichment factors that can be achieved. Sandra (2003), reported that a high enrichment factor could be achieved for analytes even with $\log K_{ow} > 5$. Zuin *et al.,* (2006), compared SBSE to membrane

assisted solvent extraction (MASE) for the determination of pesticides and benzo pyrene residues in Brazillian sugarcane juice. They found out that generally faster analysis and better recoveries were achieved using MASE, whereas greater sensitivity and repeatability were obtained with SBSE. Blasco *et al.,* (2003) used SBSE for the determination of pesticides residues in honey.

After an investigation conducted by Blasco and cowokers in 2002, they concluded that although good sensitivity was obtained, the extraction was not suitable for some polar pesticides. This means that most polar organic compounds such endocrine disruption hormones – estradiol may not be extracted by the sorptive extraction techniques. Currently only PDMS coating is commercially available. Almeida and Nogueira, (2006) reported poor recovery of less than 50% in the extaction of hormones using SBSE. In the study, SBSE and liquid desorption followed by high performance liquid chromatography with diode array detection (SBSE–LD–HPLC/DAD) were combined for the simultaneous determination of nine steroid sex hormones (estrone, 17 α-estradiol, 17 β-estradiol, 17 α - ethynylestradiol, diethylstilbestrol, mestranol, progesterone, 19-norethisterone and norgestrel) in water and urine matrices. The application of sorptive techniques to polar analytes is therefore limited.

2.4 SELECTIVE EXTRACTION

Most of the previously described extraction techniques are normally not selective for extracting an analyte from a complex aqueous sample. This normally leads to

coextraction of analytes and matrice interference which may become a problem at trace levels. As an example, most of the polar organic compounds are not easily determined by LC due to their co-elution with humic and fulvic substances present in high amount in soil and natural waters. Evidence of these compounds is usually seen as an important interferring matrix peak at the beginning of the chromatogram. Therefore, there a considerable need to have a highly selective extraction technique for the preparation and preconcentration of samples before analysis. The most common selective extraction technique solid phase extraction sorbents that are based on molecular recognition mechanisms. The benefits of selective extraction are listed below;

- Lower detection limits because the noise level would have been reduced.
- Reduced number of steps hence significant time saving.
- Significant cost savings due to time saving since clean-up steps are eliminated.
- Reproducibility of results as the interfering matrix have been removed.
- Less volume of the organic solvent used.
- Automation is simplified.

2.4.1 Immunosorbents solid phase extraction (ISSPE)

Similar to MIPs, immunosorbents phases are based upon recognition but use chemically attached mono and poly clonal antibodies rather than surface cavities. It involves the use of antigen-antibody interactions. The antibodies which are specific to

the analyte are immobilized on a solid support. Immunosorbent materials are then packed into a solid phase extraction cartridge or precolumn.

The immunosorbents phases are most selective because they are designed primarily around antigen antibody interactions that provide high selectivity and affinity. These sorbents enable selective extraction and concentration of individual components or classes of compounds popularly known as cross reactivity. Class specific immunosorbents are available for a variety of pharmaceutical, food and environmental applications (Farrė *et al.,* 2007, Delaunay – Bertoncini *et al.,* 2001). The cross-reactivity of antibodies was also exploited for the extractions of pesticides such as atrazine and terbutylazine. To recover the whole class of triazines, two antibodies were mixed in the cartridge as reported by Pichon *et al.,* (1998; 1999).

Rolcik *et al.,* (2002) developed a single-step, highly specific and easy-to-use method for isolation and purification of melatonin from complex biological matrices. Polyclonal antibodies highly specific against melatonin were raised, characterised by enzyme-linked immunosorbent assay (ELISA) and used for preparation of immunoaffinity gel. The method was successfully used for determining melatonin in human serum and turned out to be better than the non-specific solid-phase extraction. Figure 2.8 shows the comparisson of the obtained chromatograms by SPE and IS.

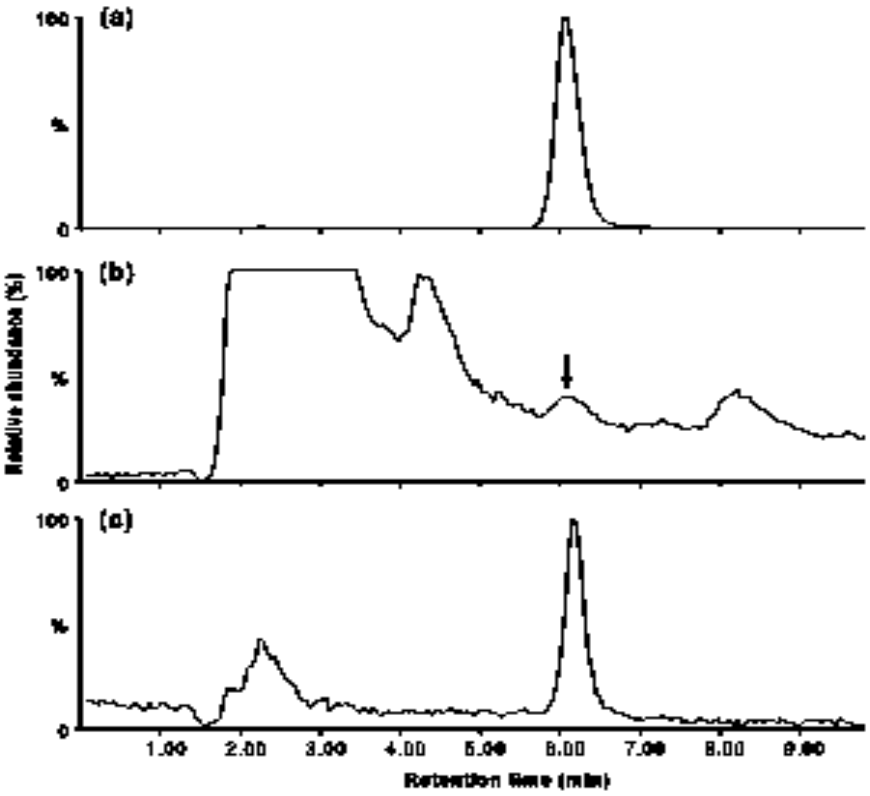


Figure 2.9: Comparison of LC–MS analyses of human serum sample processed by (b) solid-phase extraction, (the melatonin peak indicated by an arrow) and by (c) immunoaffinity purification and (a) LC–MS analysis of melatonin standard (Rolcik *et al.,* 2002).

Immunosorbents have also been developed for some veterinary drugs, such as fluoroquinolones (Holtzapple *et al.,* 1999) and corticosteroids (Stolker *et al.,* 2000). In the literature large molecules are traditionlly desorbed using chaotropic ions (those ions which favor the transfer of apolar groups to water), high salt concentration and low pH buffers. However, Farjam *et al.,* (1988) were the first to clearly demonstrate

that these conditions were not efficient to desorb low-molecular-mass organic compounds from the immunosorbent such as the β-19-nortesterone hormone.

Just like any technique, immunosorbents has its own advantages and disadvantages (Hennion, 1999). Immunoaffinity is time consuming requiring a great deal of time to make the antibody, purifying it and bonding it to a solid support. An antibody has to be developed before the IS is prepared. The need to initially develop the antibody makes it practically impossible for 'one off' analyses. The lack of homogeneity and activity in antibodies from varying commercial sources is also a disadvantage on the use of IS. An analyte is often a member of a class of compounds and antibodies are not able to distinguish between structural analogues which induces wrong estimations. Desorption of low molecular mass organic compounds from the IS is a challenge.

On the contrary, the crossreactivity of the antibody is advantageous because compounds from the same family can be extracted at the same time. It has been found out that IS provide excellent selectivity.

2.4.2 Molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers (MIPs) are synthetic polymeric materials that mimic immunosorbents. MIPs present a stable and superior binding site compared to their natural counterparts. Molecular imprinting (MI) is a useful technique for the preparation of polymeric materials with specific molecular recognition receptors. The lack of selectivity of the common SPE sorbents such as reverse phase bonded silica

and the disadvantages of immunorsobents that are outlined above led to the development of more stable and versatile MIPs. Molecular imprinted polymers (MIPs) are prepared by copolymerisation of a crosslinking agent with the complex formed from a template and polymerisable monomers. Figure 2.9 shows the schematic representation of making MIPs. The monomer and the template interact with each other by covalent or non-covalent bonding.

The covalent aproach, mainly developed by Wulff and co-workers (1972) where the template – monomer construct in solution prior to polymerisation is maintained by reversable covalent bonds. Boronic acids and Schiff bases present very suitable covalent interactions. The problem with this approach is that the interactions are too stable for splitting and too slow in the reversible interactions. Very promising interactions are non covalent ones with high association constants between the template and binding site. The most common non covalent interactions are the hydrogen bonding, ion-ion, ion –dipole and dipole-dipole. The commonly used monomer is methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) as the crosslinker. After the template has been removed from the resulting polymer matrices, using a general solvent, the resulting polymer will have specific cavities with uniform sizes able to retain selectively the template molecule by means of memory effect. Thus the retention mechanism involved is based on molecular recognition (Chapius, 2004).

MIPs have been traditionally produced mainly as bulk monoliths which are subsequently crushed and ground into a fine powder. The partial loss of the product

as fine dusts; the irregular shape particles produced and their wide size distribution have led to a search for different polymerisation methods to offset the drawbacks of the bulk polymerisation process. MIPs produced in this format are most useful for solid phase extractions (Tarkey *et al.,* 2005) and chromatography application (Andersson, 2001).

In recent years many investigators have developed new formats for MIPs that are ideally suited for specific applications, including monosized spherical beads produced by precipitation polymerisation technique for chromatography applications (Sellegreen, 2001). These spherical beads in most cases have excellent recognition properties for target molecules (Brůggerman *et al.,* 2000).

The lack of complete removal of the template molecules, even with exhaustive extraction is one of the main problems with the acceptance and usage of the MIPs. The template may interfere with the analyte especially at low analyte concentrations. One approach to overcome this problem is to use a template that is similar to the analyte of interest. Andersson *et al.,* (1997) reported the use of a brominated analogue template rather than a chlorinated molecule of interest.

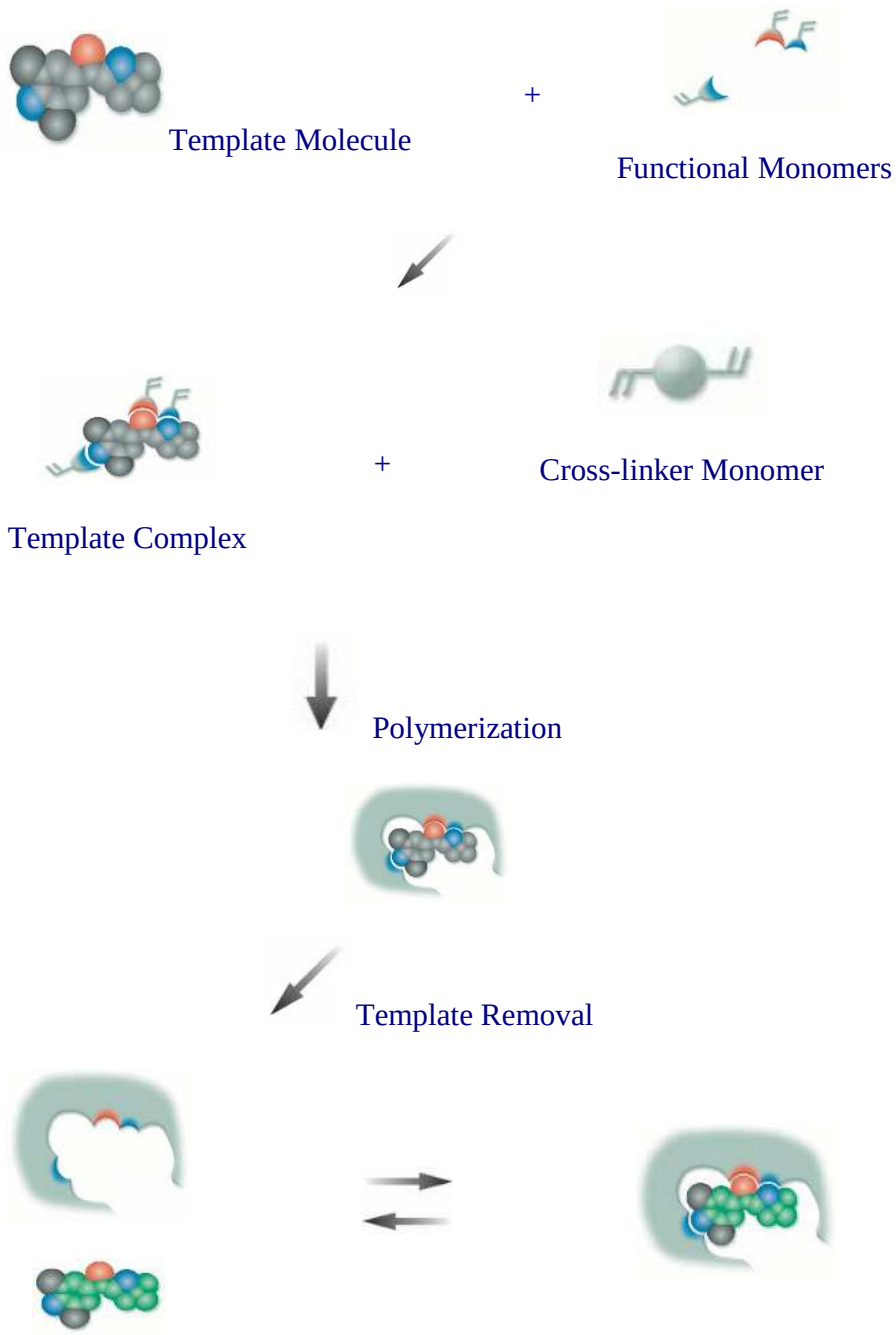


Figure 2.10: Schematic diagram showing the preparation of the MIP (www.chrysalisscientific.com accessed 10 October 2009).

Polymerisation techniques

The process of molecular imprinting starts with the selection of an appropriate imprinting approach, either covalent or non covalent. After a specific approach is selected, functional monomer(s) need to be chosen so that a good template-monomer complex can guide the formation of the template-specific recognition sites. Other components such as the cross-linker and the porogen are also chosen. The imprinting efficiency is enhanced when the polymerisation reaction is performed in a non-protic solvent as apolar as possible. The most widely used solvents are acetonitrile, chloroform, dichloromethane and toluene (Kaabi and Pichon, 2007). Below is a summary of some of the polymerisation techniques:

Bulk polymerisation

The classical way of preparing MIPs is by bulk polymerisation (Arshady and Mosbach, 1981; Vlatakis *et al.*, 1993; Wulff *et al.*, 1977). It consists of the synthesis of a block monolith polymer followed by grinding to particles with a wide range of size distribution. Extensive sieving is necessary to achieve a more narrow size distribution. Usually, these particles are sieved to yield particular sizes with nominal diameter in the 25-35 µm range (Kaabi and Pichon, 2007). Turiel *et al.,* (2003) and Cacho *et al.*, (2003) prepared the MIP using propazine as a template for the extraction of triazine herbicides. Several other reseachers (Bjarnasson *et al.*, 1999; Sellergren, 1994; Pichon and Haupt, 2006; Ferrer *et al.*, 2000) reported the preparation of bulk

polymers. Bulk polymers for 17 β-estradiol were synthesised by Wei *et al.,* (2006) in 6 ml of acetone as the porogen (the solvent were polymerisation takes place).

The particles are then packed into disposable cartridges and used as a SPE sorbent. Although it is easy to prepare, bulk polymerisation is time consuming, labour intensive and wasteful because only 30-40% of the ground polymer is usually recovered as usable material. Moreover, the particles are far from ideal because of their irregular shape and wide size range. They are packed poorly in columns and created large void volumes, which may be a problem when the MIP is coupled on-line to LC. These significant drawbacks limited the suitability of MIPs especially for HPLC separations and binding assays but they don't limit their use in SPE especially when performed in an off-line procedure (Andersson, 2001).

Precipitation polymerisation

Precipitation polymerisation has been used to produce mono-disperse microsphere MIPs. The formation of highly crosslinked microspheres takes place in an excess amount of reaction medium. The volume is typically 2-10 times higher than the volume normally used in bulk polymerisation (Kaabi and Pichon, 2007). The mechanism for particle formation and growth in precipitation polymerisation has been proposed to resemble that of dispersion polymerisation, except that the particleas are stabilized against coagulation by their rigid, crosslinked surfaces, rather than by added stabilisers (Li and Stöver, 1993).

As the polymerisation proceeds, the growing polymer chains become insoluble in the liquid phase so they precipitate and the solution becomes heterogeneous. Micro- and nanospheres can be generated when accurate control of the parameters governing the precipitation polymerisation are achieved. There is no need for grinding and precipitation after polymerisation. Therefore the preparation of molecularly prepared microspheres is less time consuming when compared to the conventional bulk polymerisation. The yield is often greater than 85%. However the presence of the print molecule affects the polymer mophology (Tamayo *et al.,* 2005 and Cacho *et al.,* 2004). Cacho *et al.,* (2004) reported that particles obtained are colloidal and slightly irregular in shape.

Precipitation polymerisation was used for the production of MIPs for different phenyurea herbicides such as fenuron (Tamayo *et al.,* 2003) and propazine (Cacho *et al.,* 2004). Carabias-Martinez *et al.,* (2005) prepared a propazine MIP by precipitation polymerisation for the extraction of triazine herbicides and some of their hydroxylated and dealkylated metabolites from river water. Extraction recoveries higher than 75% were obtained for chloro and methylthio-triazines when extraction was performed in organic medium (toluene). Low extraction recoveries were obtained when extraction was performed in aqueous medium.

Meng *et al.,* (2005) employed MIPs in the removal of estrogenic pollutants from contaminated water. The MIPs microspheres sized from 1 to 2 µm were synthesised using α-estradiol as the template and acetonitrile as the porogen. The prepared MIP exhibited significant binding affinity toward other related estrogenic compounds such

as 17 β-estradiol, diethylstilbestrol, estriol and estrone. It was also shown that the MIPs were sufficient to remove EDCs present at several nanograms per liter from untreated environmental waters.

Suspension polymerisation

Suspension polymerisation is a heterogeneous polymerisation method for the production of spherical beads. This method has been employed for the preparation of molecular imprinted polymer beads, using both aqueous and non-aqueous continuous phases. In this process, the organic-based polymerisation mixture is suspended as droplets into an excess of a continuous dispersion phase by an agitation method. The polymerisation proceeds by a free radical mechanism in each droplet. As compared to the bulk polymerisation, the suspension polymerisation is performed at a higher molar concentration of template and monomers to compensate the partial loss of the reagents in the dispersion medium (Lai and Wu, 2003). This method gives larger beads and a broader distribution in particle size, although the latter can be controlled to some extent by optimizing the reaction conditions (Yan and Ramström, 2005).

Meyes *et al.,* (1996) reported the suspension polymerisation in perflurocarbon fluids which are preferred to water because they are inert, stable and largely immiscible with organic compounds and the template. They did not disrupt the interactions between the functional monomers and the template. Lai and Wu, (2003) prepared two types of trimethoprim (TMP) MIPs by both bulk and suspension polymerisation. The MIP particles based on bulk polymerisation were most selective. Suspension

polymerisation is not very common compared to bulk and precipitation polymerisation methods because the medium in suspension is either polar water or expensive perflurocarbon fluids (Kaabi and Pichon, 2007, Mayes and Mosbach, 1996).

Multi-step swelling polymerisation

Multi-swelling polymerisation technique is a method of suspension polymerisation in which water could be used as the continuous phase. It was developed in order to control the size distribution, the shape of the MIP particles and to decrease the amount of waste material. Haginaka's group widely used this two step swelling method in the synthesis of polymer beads (Tamayo *et al.*, 2007).

Mono-dispersed spherical polymer particles over a size range 5-100 µm are produced. It involves the production of uniformly sized polystyrene beads called seeds of about 1 µm diameter. These seeds are used for the two subsequent swelling steps. After the first step, which the seeds are swollen using an aqueous micro-emulsion of a free radical initiator and a highly water insoluble solvent such as dibutyl phthalate. The swollen particles dispersion is added to a second aqueous dispersion of the polymerisation mixture. The mixture is stirred until the droplets of the polymerisation mixture are adsorbed on the swollen particles. The polymerisation then follows the second swelling stage. After 24 hrs, the polymer beads are then washed and their size would have increased 5 – 1000 times larger.

Haginaka's group (1994) was first to report aqueous two step swelling applied to molecular imprinting of diaminophthalenes. A two step swelling approach was used by Kubo *et al.,* (2006) for the preparation of domoic acid MIP. Watabe *et al.,* (2006) used the same technique for the preparation of 17 β-estradiol MIP. A one-step swelling approach was reported by Liu *et al.,* (2007) to prepare a MIP for the selective extraction of metsulfuron-methyl from drinking water.

Application of MIPs to solid phase extraction

MIPs have been used as sorbents for the pre-concentration, cleaning and selective extraction of the target analyte of samples. Molecularly imprinted solid phase extraction (MISPE) is based on conventional SPE procedures. In MISPE, the MIPs can be packed in a cartridge between two frits and be used off-line. They can also be packed in a small column (20-50 mm x 2-4 mm i.d.) to be coupled on line with LC (Caro *et al.,* 2003, Koeber *et al.,* 2001 and Watabe *et al.,* 2005). The steps involved in MISPE are conditioning, loading, clean up and elution. These steps were discussed earlier in SPE. After conditioning, the loading (extraction) step can be performed either in organic or in aqueous medium (Ferrer *et al.,* 2000, Chapius *et al.,* 2003), followed by a washing step which can be used to remove interfering compounds. It has been largely demonstrated that the MIP offer the highest selectivity when analytes are dissolved in the solvent used for the MIPs preparation (porogen) (Ferrer *et al.,* 2000, Chapius *et al.,* 2003). Desorption of the analytes is achieved by percolating a solvent able to break the interaction between the MIP and the analytes.

Chapius *et al.,* (2004), developed an extraction method in which the selectivity of the MIP prepared using ametryn as the template was compared with an imminosorbent (IS) based on anti – triazine polyclonal antibodies immobilised on silica. It was found out that the selectivity of the MIP was comparable to the selectivity of IS as shown in the Figure 2.10.

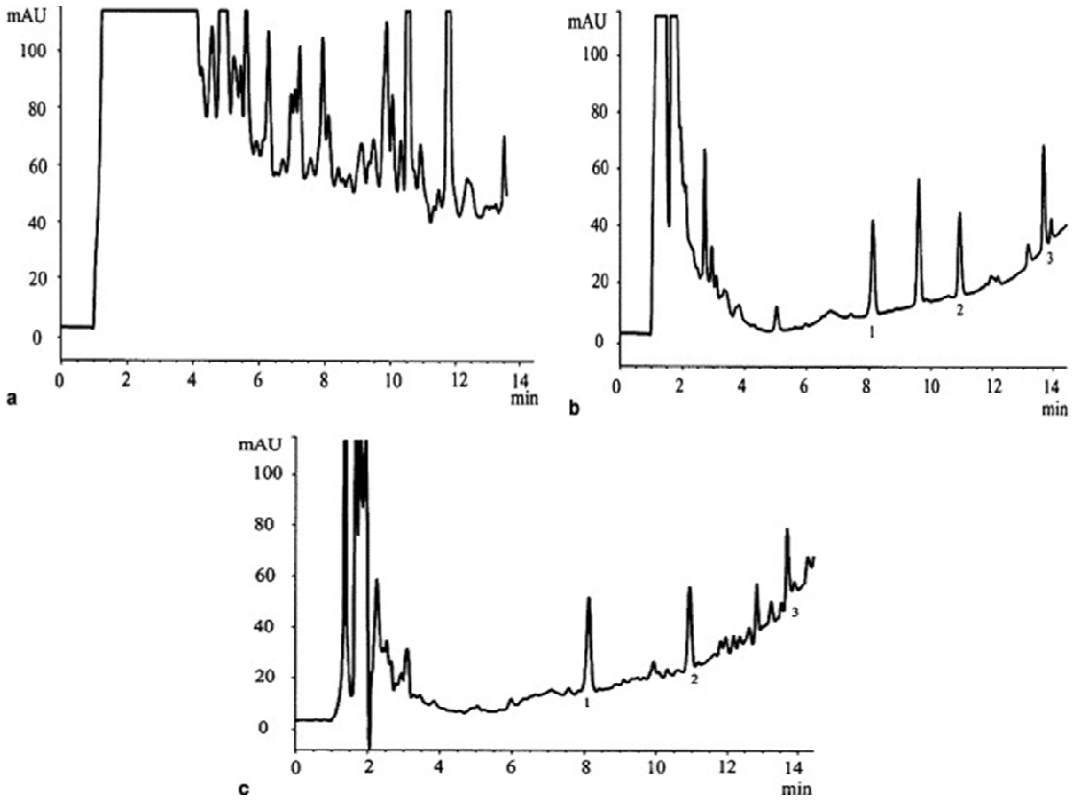


Figure 2.11: Chromatogram obtained after the injection of a soil extract containing 20 ng g^{-1} of triazines: (a) without and (b) with a clean-up step on the MIP; and (c) on the anti-triazine immunosorbent. Peak assignment: (1) atrazine; (2) simazine; and (3) terbutylazine (Chapius *et al.,* 2004).

MIPs were also employed for the extraction of fluoroquinolones from soil extracts as reported by Turiel *et al.,* (2007). Percolation of the sample was perfomed in porogen (methanol), the washing solution consisted of methanol and acetic acid mixture. Without MISPE, the identification and quantification of fluoroquinolones were impossible. However by using MISPE, the identification and quantification of all fluoroquinolones was easily accomplished because cleaner extracts were obtained.

Besides MIPs being used for SPE, they are also exploited in many applications that include (Vlatakis *et al.,* 1993; Headborg *et al.,* 1993; Hong *et al.,* 1997; Piletsky 1999; Kempe, 1996; Yu and Mosbach, 1997; Fisher *et al.,* 1991; Ramstrom *et al.,* 1995; Meng *et al.,* 2005).

- enzyme mimics in catalysis.
- recognition elements in biosenors.
- membrane separation technology.
- product purification.
- wastewater purification.
- selective filtration.
- chromatographic separation of enantiomers.
- drug development.

MISPE are cheaper and easier to prepare when compared to IS. One weakness of IS is that they provide a challenge on handling and their stability is a cause of concern

whereas MIPs are easier to handle and very stable. Excellent selectivity and preconcentations can be obtained by using MIPs in SPE.

The lack of removal of the template even after exhaustive extraction technique is the main problem with the use of MIPs. MIPs works best in organic solvents making it difficult to extract aqueous samples directly. The other disadvantage of MIPs is the slow binding kinetics for some compounds.

2.4.3 Multimodal and Mixed –mode extraction

Most SPE sample preparation involves the use of a single mechanism. However when additional selectivity is needed to isolate a single analyte, multimodal can prove useful. Multimodal SPE refers to the intentional use of more that one retention mechanism. Mixed mode sorbents that are available use both primary and secondary mechanism for selective retention of analytes. Mixed mode retention were observed during the solid phase extraction of basic drugs using a reversed-phase silica containing residual silanols at sample pH where both entities are ionized. In some cases mixed-sorbents allow the extraction and the clean-up of environmental and biological matrices in the same sequence.

In the extraction of drugs, the most popular cartridge or disk format contain octyl chains and cationic-exchange groups. As in all SPE techniques, the sorbent is equilibrated. The sample pH is adjusted such that the analytes are in their neutral or negatively charged forms. During sample loading analytes of interest (drugs) and other matrix interferences are retained by hydrophobic interactions. The next step is

to protonate the amino group of the drug by acid washing. This makes the drug to be strongly bound by ionic interaction compared to other interfering matrix components that are bound by hydrophobic interactions. To elute these interfering compounds, the catridge is washed with a weak organic solvent such as methanol and the drugs bound by stronger ionic interactions will remain retained. Final desorption is achieved by a basic solution that will break the ionic interactions and therefore selectivity will be achieved.

In a SPE review by Hennion (1999), the use of PS-DVB-based mixed mode resin that contains C_{18} and sulfonated cation-exchange groups were shown to be efficient for isolating triazines and basic drugs, combining hydrogen bonding, cation-exchange and Van der Waals interaction.

Raisglid and Burke (1997) used two different adsorbents to isolate different molecular species. They used a layered amino sorbent over a C_{18} sorbent to remove humic acid when extracting pesticides from water samples. The amino sorbent strongly bound the humic molecules that have acidic or phenolic groups while the pesticides passed through the amino layer were sorbed by the hydrophobic C_{18} layer. The pestcides could then be eluted by an organic layer and analysed.

Aproaches to Multimodal SPE

There are two main approaches to multimodal SPE shown in Figure 2.11 below. These are serial approach and single cartridge format. In a serial approach, two SPE cartridges are connected in series (Figure 2.11a). One catridge is packed

with a different sorbent compared to the other. As an example Tanibe and Kawata (2003) reported a method they developed for the simultaneous extraction of 5 triazines and 12 degradation products in environmental samples using a graphitised carbon and styrene-divynylbenzene copolymer. In a single cartridge format, there are two or more functional groups which can interact with the analyte (Figure 2.11b).

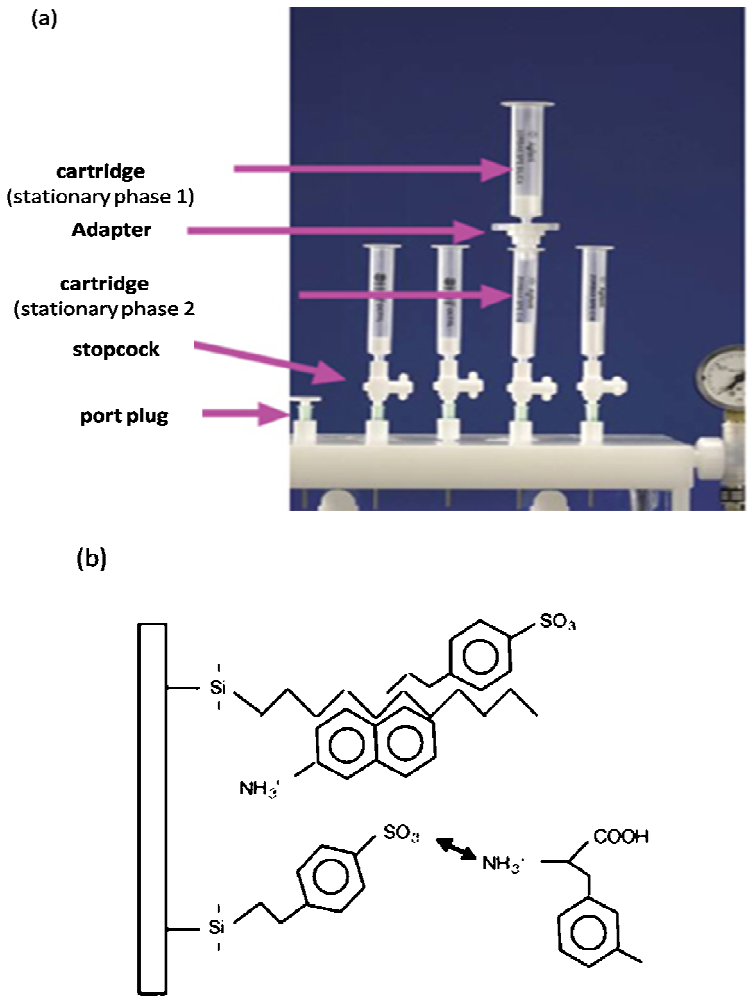


Figure 2.12: Aproaches to mixed mode extraction (a) serial approach (b) catridge containing two functional groups (Majors, 2007)

2.4.4 Restricted access media (RAM)

This is a special class of SPE sorbents used for the direct injection of biological fluids such as plasma, serum or blood. Only analytes of low molecular mass have free access to the binding sites at the inner surface and thus can be retained and selectively extracted. RAM sorbents combines size exclusion of protein and other high molecular mass matrix components with simultaneous enrichment of low molecular mass analytes at the inner pore surface. The interaction sites within the pores are accessible to small molecules only and analytes are retained by conventional retention mechanisms such as hydrophobic or electrostatic interactions. Various RAM sorbents are available with the different surface chemistries.

RAM phases that are most popular are the dual – mode porous packings that are characterised by hydrophilic diol – type functionality on the outer surface of silica particles and reverse phase (hydrophobic) alkyl chains attached to the interior pore surfaces. The outer hydrophilic surface with minimal interactions with proteins combined with small pores of the packing that exclude them cause the proteins to be eluted unretained, while small drugs and metabolites pass into the pores and are retained by hydrophobic interactions with alkyl bonded phases. Figure 2.12 shows the diagram of a RAM.

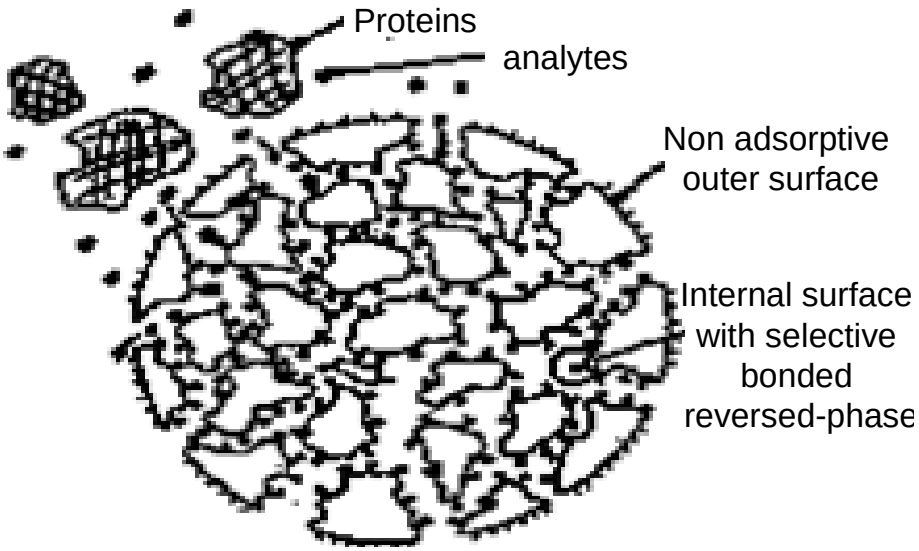


Figure 2.13: A diagram of dual RAM phases

Doerge *et al.*, (2000) reported on line sample clean-up of rat serum using a RAM trap catridge (Diazem C_8) in the analysis of isoflavones, genistein and diazem. A study by van der Hoeven *et al.*,(1997) described the analysis of cortisol and prednisolone in plasma and arachidonic acid in urine samples by LC-MS with an on-line SPE using a restricted access media precolumn. The only offline sample pretreatment step required was centrifugation to remove particulate matter. A precolumn packed with 25 µm C_{18} alkyl diol silica was coupled to LC without transfer loss. Another selective multicolumn LC method was described by Mangani and coworkers (1997), for the analysis of six cardiovascular drugs in serum. The method consisted of an on-line sample clean up using a pinkerton GFF2 restricted access precolumn, followed by chromatographic separation employing a C_{18} column, and front cutting to perfom a chiral separation of pindolo enantiomers on a second LC system. After filtration

spiked and non-spiked serum samples were loaded onto the precolumn and the washing conditions were optimised in order to have an efficient removal of matrix interferences using minimum of washing solvent with retention of all the analytes on the precolumn. Figure 2.13 shows the chromatogram corresponding to the on line analysis of a drug-free serum, the standards and the spiked serumn. No intereferences were detected. In another study a RAM utilising a not so selective normal phase was employed to selectively extract methyl oleate from a partially selective semipemeable membrane device extract (Gustavson *et al.*, 2000).

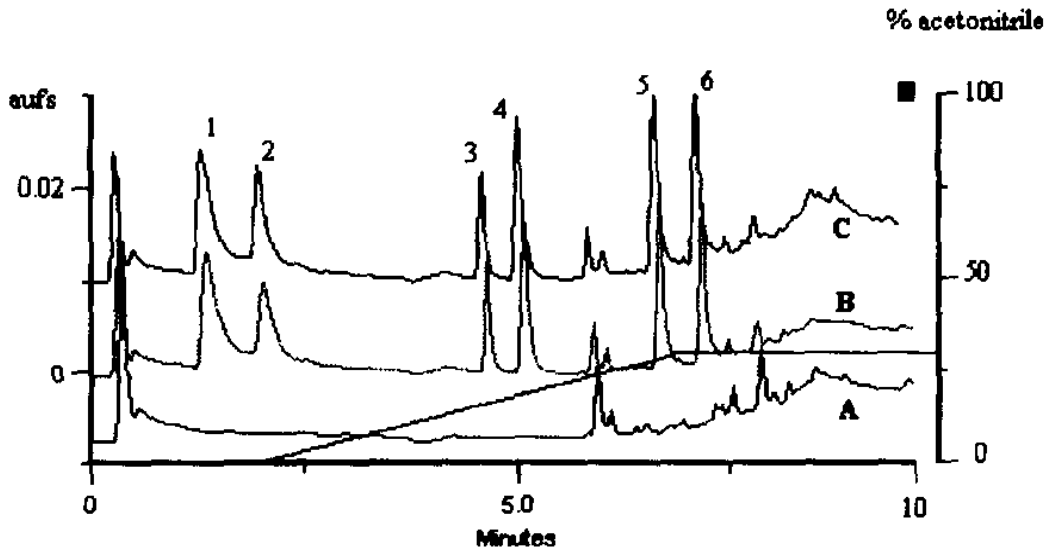


Figure 2.14. On line clean up and analysis of cardiovascular drugs in serum with HPLC system. (A) Drug-free serum, (B) standards in buffer and (C) spiked serum. Precolumn packed with a Pinkerton GFF2 restricted access sorbent. Peaks; 1 = lodocaine, 2 = pindolol, 3 = metaprolol, 4 = oxyprenolol, 5 = diltiazem, 6 = verapamil (Mangani *et al.*, 1997).

The advantages RAM are summarised as follows: it enables direct injection of untreated biofluids, it eliminates other clean-up steps such as precipitation of proteins in serum and blood plasma and lastly it does not allow irreversible interaction with macromolecule sample components (Boos, 1999).

The disadvantages of RAM are summarised as follows: it has a reputation of fouling with repeated injections of straight biological fluids and complex matrixes, RAM is not very selective among the small molecules although the inner surface can be modified to enhance the selectivity and in some cases protein precipitation may occur in RAM.

2.5 COMBINATION OF EXTRACTION TECHNIQUES TO INCREASE SELECTIVITY

Other approaches to increase selectivity is to combine two or more sample preparation techniques especially a non selective with a selective one. This approach is fast gaining recognition in sample preparation techniques. The following combination techniques are examples of the such found in literature. Chromatograms and electropherograms are shown where necessary in order to compare the selectivity without and with combination.

2.5.1 Solid phase extraction – molecularly imprinted polymers solid phase extraction (SPE –MISPE)

MIPs works better if the extraction is performed in an organic liquid. Therefore SPE before MIPSE has been widely employed to transfer the analytes from the aqueous phase to the organic phase. Solid phase extraction, especially C_{18} are known to lack selectivty, hence a clean-up step utilising a MIP is believed to produce clean extracts that may result to lower detection limits.

Pap *et al.*, (2002) used a terbutylazine MIP as a clean-up step after C_{18} SPE of river samples. The procedure involved percolating SPE (C_{18}) cartridge with 500 ml water sample containing 0.5% methanol. Dichloromethane was used to desorb the bound triazines directly on the MIP for a clean-up step. Methanol was used to elute the bound triazines from the MIP. Clean chromatograms were obtained. In a study conducted by Carabias-Martínez *et al., (*2005), propazine MIP prepared by precipitation polymerisation was used as a clean-up step after a LiChrolut EN SPE preconcentration, resulting in clean chromatograms. Bjarnasson and coworkers, (1999) conducted some work where the triazine MIP was coupled to the SPE (C_{18}) to extract triazine herbicides from urine and apple extracts. The chromatograms (Figure 2.14) obtained were cleaner than those only using C_{18} column.

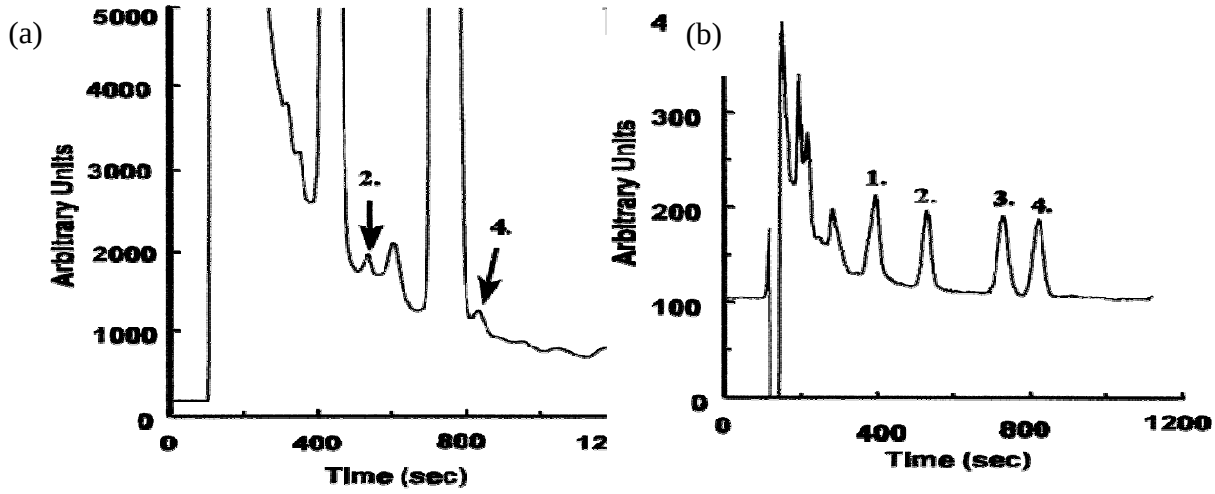


Figure 2.15: Extractions of triazines from a highly contaminated sample (a) SPE with MIP omitted, and (b) SPE (C_{18}) - MIP extraction. (Bjarnasson *et al.,* 1999).

2.5.2 Supported liquid membrane – solid phase extraction (SLM – SPE)

The application of supported liquid membrane (SLM) extraction prior to solid phase extraction (SPE) was investigated for atrazine enrichment and clean up from complex matrices such as fruit juices (Khrolenko *et al.,* 2002). In comparisons to SPE extraction from juice samples, the application of SLM – SPE combination provided much cleaner extracts as shown in the electropherograms (Figure 2.15). The combination also provides the possibilities of lowering the detection limit to 30 µg l^{-1} using capillary electrophoresis as the separation technique. The use of SLM-SPE lowered the quantification limit from 1000 µg l^{-1} (for SPE only) to 50 µg l^{-1}. In this report, the acceptor phase of the SLM consisted of 10 ml 0.5 M H_2SO_4. After the extraction it was neutralised by NaOH prior to SPE on a Speedisk C_{18} disk.

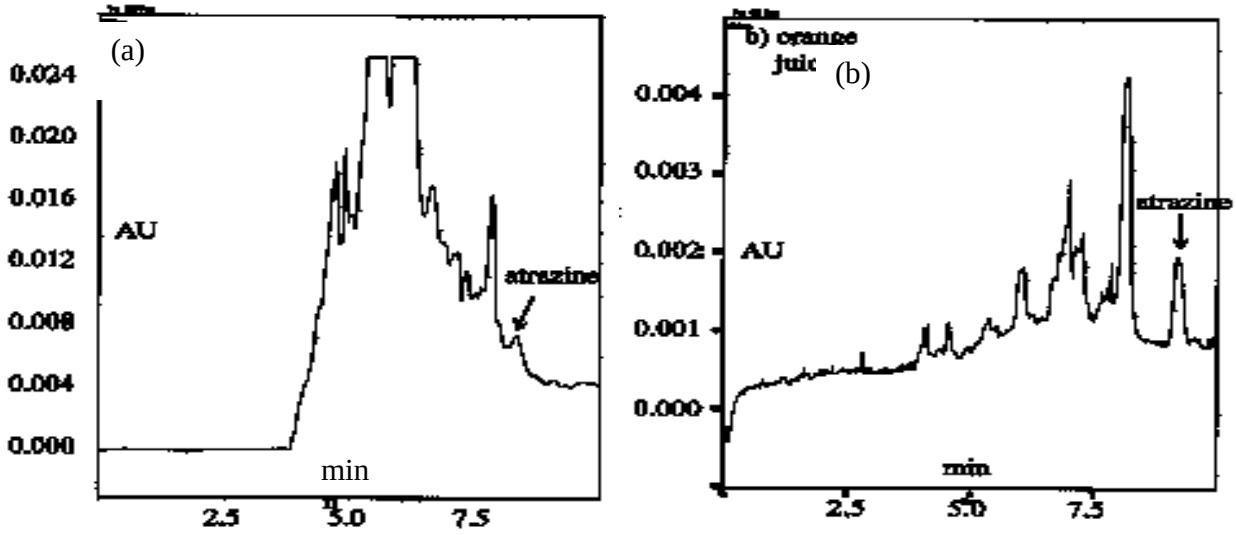


Figure 2.16: Electropherograms of atrazine in orange juice after, (a) SPE only and (b) SLM-SPE extractions. Taken from Khrolenko *et al.,* (2002).

2.5.3 Restricted access media - molecularly imprinted polymers (RAM – MIPs)

A number of studies have reported RAM-MIPs extraction method (Haginaka *et al*., 2000, Sambe *et al.*, 2007, Boos *et al.,* 2001). RAM have unique characteristics which can exclude macromolecules, such as serum proteins, and allow small molecules such as drugs and their metabolites to penetrate into binding sites, and have been used for direct injection of biological samples. A RAM-MIP is prepared by multi-step swelling polymerisation as described previously and hydrophilisation of the external layer. One study on this combination was performed by Haginaka *et al.,* (2000) on rat plasma where a RAM polymer was imprinted to produce a RAM - MIP material. In another study a RAM – MIP for irgarol was prepared followed by in situ hydrophilic surface modification using glycerol dimethacrylate as hydrophilic monomers (Sambe

et al., 2007). The RAM - MIP was applied to selective pretreatment and enrichment of methylthiotriazine herbicides, simertryn, ametryn and prometryn in river water followed by their separation and UV detection via column-switching HPLC.

Koeber *et al.,* (2001) reported another combination of RAM and MIP. The combination of a RAM and a MIP allowed a selective sample preparation to be achieved in the on-line mode. The cleaned and enriched extract was subsequently eluted to an HPLC column and analyzed by LC-MS. The chromatograms below (Figure 2.16) shows enhanced selectivity after the combination.

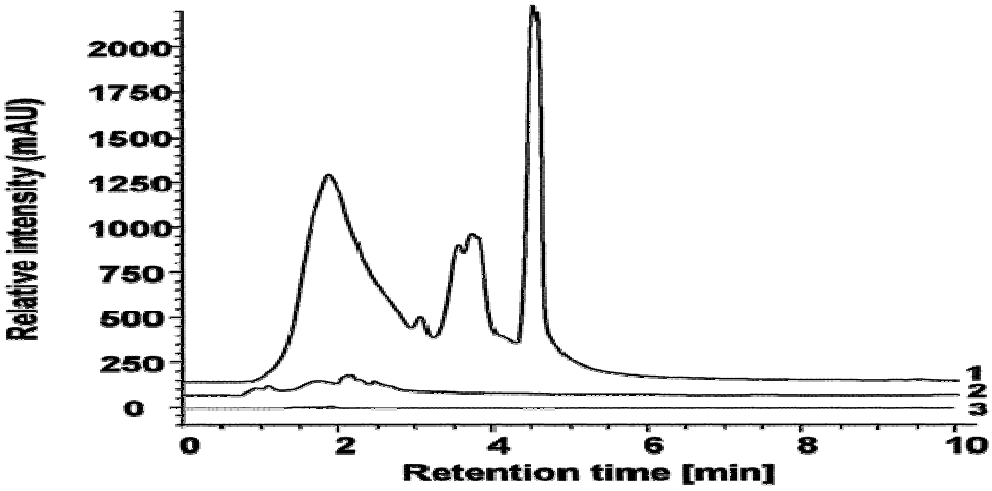


Figure 2.17: The reduction of interfering matrix components (1) HPLC column only, (2) RAM – LC coupling, and (3) RAM – MIP –LC Coupling (Taken from Koeber *et al.,* 2001).

2.5.4 Semi - permeable membrane devices - normal phase based RAM (SPMD – NP RAM)

Semi-permeable membrane devices (SPMDs) have been used extensively as passive samplers for monitoring hydrophobic contaminants in aquatic systems (Huckins *et al.,* 1993 and Lebo *et al.,* 1995). SPMD is polyethylene tubing that is sealed at both ends after filling it with an extraction liquid and submerged into an aqueous sample that is to be extracted. The SPMD effectively concentrate non-selectively hydrophobic compounds from a large volume of water via passive partitioning into the membrane from the surrounding water.

Gustavson *et al.,* (2000) combined the benefits of SPMD and a not so common normal-phase RAM for the selective removal of methyl oleate during the analysis of polynuclear aromatic hydrocarbons. Methyl oleate which is often found in the final dialysate of the standard SPMD concentrate emanates from the polyethylene tubing impurities. This causes problems in the analytical stage because of co-elution with target compounds. The methyl oleate was retained by the outer hydrophilic ligands whilst the PAH passed through.

2.5.5 Immuno - SLM

This technique was first reported by Thordarson *et al.,* (2000). Emnéus' research group at Lund University has been instrumental in the development of this technique. The SLM consists of two aqueous phases separated by a hydrophobic polymer impregnated with an organic liquid. The extraction involves the partitioning of

neutral compounds into the organic phase. From the membrane re-extraction takes place into the acceptor phase containing antibodies specific for the target compounds, hence antibody-antigen complex are formed. The formed immunocomplex was quantified on-line, using a fluoresce in flow immunoassay in a sequential injection analysis (SIA) set up. The notable shortcoming noticed in this work was that when working with polyclonal antibodies against small molecules such as 4-NP, is that cross reactivity tends to be somewhat high.

Tudorache *et al.,* (2004) aplied the Immuno-SLM extraction of atrazine from tap water, river water and orange juice. Tudorache and Emnéus, (2005) extracted 2,4,6-trichlorophenol (2,4,6-TCP) from spiked river samples using this combination technique. An improved selectivity was obtained but the sensitivity was low.

CHAPTER 3: SEPARATION TECHNIQUES

Chromatographic separations of mixtures of various organic compounds are based on their distribution between a stationary and a mobile phase, which are present in a chromatographic column. There are several types of chromatographic separation techniques which includes high performance liquid chromatography (HPLC), gas chromatography (GC), and ion chromatography (IC).

All chromatographic systems contain a stationary phase, mobile phase and the detector. The stationary phase provides a retarding force which holds back molecules with which it interacts. The mobile phase moves across the column, in effect washing (eluting) compounds at a different rate. These differences are based on properties such as the boiling point, the polarity, the electric charge (for ionic compounds), the size of the molecule, and so forth. At the column outlet there is a system for detecting and measuring the quantity of each component, therefore a quantitative determination of the separated components is achieved.

Both HPLC (coupled to detectors such as UV, MS etc) and GC (coupled to FID, MS detectors) are commonly used as determination techniques in the analysis of organic compounds such as triazines (Pacáková *et al.,* 1996).

3.1 HIGH PERFOMANCE LIQUID CHROMATOGRAPHY (HPLC)

High-performance liquid chromatography (HPLC) uses liquid mobile phase to separate compounds that are dissolved in solution. Chemical separations can be

accomplished using HPLC by utilizing the fact that certain compounds have different migration rates in a given particular column and mobile phase. Components injected in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. The stationary phase in liquid chromatography can be solid (e.g. silica) or liquid bonded onto silica particles (e.g. C_{18}). The mobile phase and stationary phase are immiscible to avoid removal of the stationary phase. HPLC has many applications including separation, identification, purification, and quantification of various compounds (Knox *et al.*, 1989).

Separation in HPLC is based upon the relative abilities of the stationary phase to trap analytes and allow them to elute over time. As molecules of the sample components enter the column, it can be either be adsorbed on the stationary phase or remain in the mobile phase. A strongly adsorbed sample component spends a greater proportion of its time within the column on the stationary phase than does a weakly adsorbed component. Consequently, the retention time or volume increases as the amount of adsorption on the stationary phase increases (Braun, 1987). There are several types of stationary phases used in the HPLC analysis, but the most commonly used are the normal phase and reversed phase sorbents (Winkler, 2001).

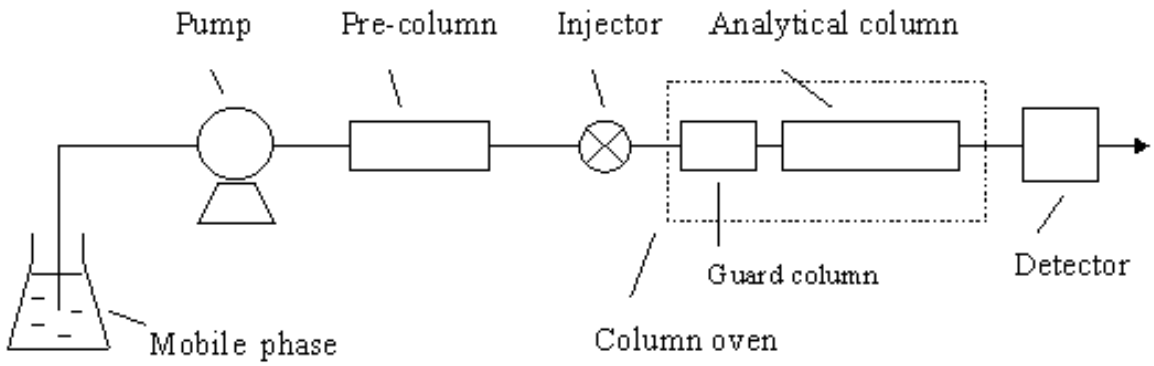


Figure 3.1: Schematic representation of the HPLC

3.1.1 Different modes of HPLC

Normal phase chromatography

Normal phase HPLC separates analytes based on polarity and the interactions between the analyte and the stationary phase is purely adsorptive. This type uses polar stationary phase (amino, cyano or silica) and a non polar mobile phase (eg dichloromethane). It is used when an analyte of interest is relatively polar. This type of HPLC is not commonly used even for the determination of triazines. Equilibration between mobile phase and stationary phase takes long time and therefore cannot perform gradient elution.

Reverse phase chromatography

This consists of a non-polar stationary phase (C_{18}, C_8 etc) and a polar mobile phase (methanol – water mixture). It is used when an analyte of interest is non polar.

Reverse phase operates on the principle of hydrophobic interactions which results from the repulsive forces between a relatively polar solvent, the relatively non polar analyte, and the non polar stationary phase. Reverse phase chromatograph has been widely used in analytical techiniques.

Reverse phase sorbents have been widely used in analysis of triazine compounds (Cacho *et al.*, 2004, Carabias-Martinez *et al.*, 2006, Ferre *et al.*, 2000). Cacho *et al.*, (2004) used a C_{18} column for the analytical separation of triazine herbicide prior to detection using a photo diode-array detector.

3.1.2 HPLC detectors

The detector for an HPLC is the component that emit a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is connected to the end of the column in order to detect the compounds as they elute from the column. There are many types of detectors that can be used in HPLC. Some of the more common detectors include: refractive index, ultra-violet, fluorescent, radiochemical, electrochemical, near-infra red, mass spectroscopy, nuclear magnetic resonance, and light scattering (http://kerouac.pharm.uky.edu accessed 17July 2009).

Ultraviolet (UV) detectors measure the ability of a sample to absorb light at one or more wavelengths. Light scattering detectors nebulize the effluent, vaporize the solvent, and then detect droplets in a light scattering cell. Electrochemical detectors measure the current from the oxidation/reduction reaction of an analyte at a suitable electrode. Radiochemical detectors use tritium or carbon-14 to detect the fluorescence

associated with beta-particle ionization. Mass spectrometry detectors ionize a sample and use a mass analyzer to detect the ion current. Nuclear magnetic resonance detectors irradiate nuclei that are placed between the poles of a strong magnet. The radiation is absorbed, the parallel nuclei enter a higher energy state, and each atom produces a spectra specific to its location and chemical composition. Some HPLC detectors measure the change in the refractive index of the column effluent passing through the flow cell. Others detect the fluorescence that occurs when compounds are excited by shorter wavelength energy and emit higher wavelength radiation.

The most commonly utilised detector in HPLC work is UV~VIS. UV absorption detectors respond to those substances that absorb light in the range 180 to 900 nm. Most triazines exhibit absorption maxima in aqueous solutions around 220 to 225 and/or 255 nm, while their hydroxyl derivatives absorb at lower wavelengths (around 215 nm). Three examples of UV detectors are fixed wavelength, which measures at one wavelength, usually 254 nm, variable wavelength which measures at one wavelength at a time, but can detect over a wide range of wavelengths and diode-array which measures a spectrum of wavelengths simultaneously. The diode array has an advantage in that it is used for further identification. The UV spectrum of any compound is unique though compounds of the same family tend to have similar spectrum. These can be differentiated on the basis of retention time.

The Beer-Lambert Law gives a quantitative relationship between the light absorbed as it passes through the cell containing sample and the concentration of the analyte.

$$\mathbf{A = \varepsilon bc} \qquad (3.1)$$

Where **A** is the absorbance and has no units, since $A = log_{10} P_0 / P_.$, **ε** is the molar absorbtivity with units of l $mol^{-1}cm^{-1}$, **b** is the path length of the sample - that is, the path length of the cuvette in which the sample is contained in cm, **c** is the concentration of the compound in solution, expressed in mol l^{-1}.

3.2 GAS CHROMATOGRAPHY

In gas chromatography, gaseous analytes are transported through the column by a gaseous mobile phase, called carrier gas. The stationary phase in this type of chromatography is usually a non-volatile liquid bonded onto a solid but sometimes it can be only a solid (Harries, 1987). The schematic diagram of gas chromatograph is shown in Figure 3.2.

There are two general types of column, packed and capillary (also known as open tubular). Packed columns contain a fine solid support coated with a non - volatile liquid stationary phase; or the solid itself may be the stationary phase. Packed column are useful for preparative separations, when a great deal of stationary phase is required, or to separate gases that are poorly retained (Harries, 1987). Narrow open tubular columns are commonly used because they provide higher resolution than wider open tubular columns which requires higher pressure to operate (Scott, 2007). Capillary columns are superior to packed columns because the mass transfer coefficient contribution to band broadening due to multiple path lengths is eliminated.

This is because the stationary phase is in the sides of narrow bore. However the capacity is much lower compared to packed columns.

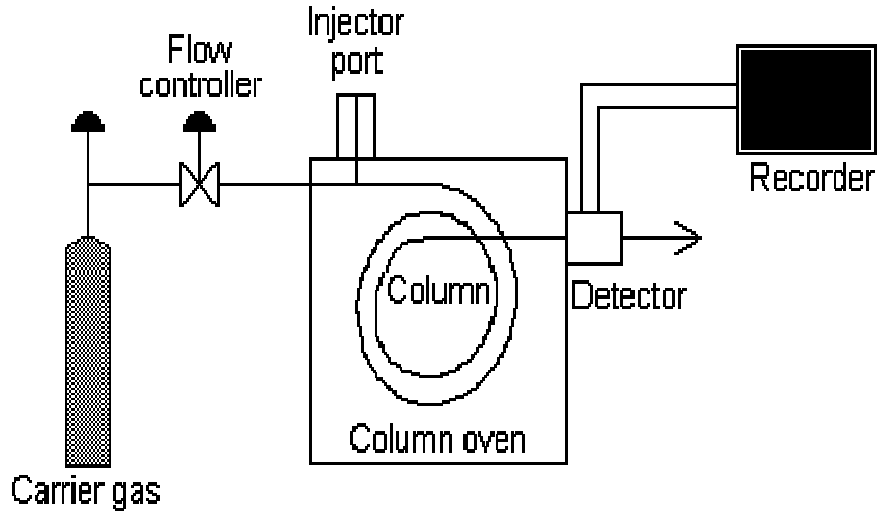


Figure 3.2: A schematic diagram of gas chromatograph (McCarthy, 2001).

3.2.1 Detectors in GC

Mass Spectrometry

The GC-MS is composed of two major building blocks: gas chromatograph usually a capillary column and the mass spectrometer. The differences in the chemical properties between different molecules in a mixture will separate the molecule as the sample travels the length of the column. The molecule will be eluted with different retention time from the gas chromatograph and then that allows the mass spectrometer to ionise and detect the molecule separately. The mass spectrometer

does this by breaking each molecule into fragments and detecting these fragments using their mass to charge ratio.

Flame ionisation detector

The flame ionisation detector (FID) is used to measure concentrations of hydrocarbons within a sampled gas. The presence of hydrocarbons is detectable by burning the sample gas in an air-hydrogen flame. Burning just pure hydrogen with air produces only trace amounts of ionisation. The presence of hydrocarbons in the sample, when burnt with an air-hydrogen mix causes high levels of ionisation. The ionisation occurs as a result of the carbon atoms present in the sampled gas. The level of ionisation is proportional to the number of carbon atoms within the sample (Fackrell, 1980). This relationship is used for the quantification by running standard samples. The FID is the commonly used detector in GC. The diagram of FID is shown in Figure 3.3.

s-Triazines can be detected with FID, but a more sensitive and selective response is obtained when using the nitrogen-phosphorous detector (NPD) because of the presence of nitrogen atoms in the analyte molecules

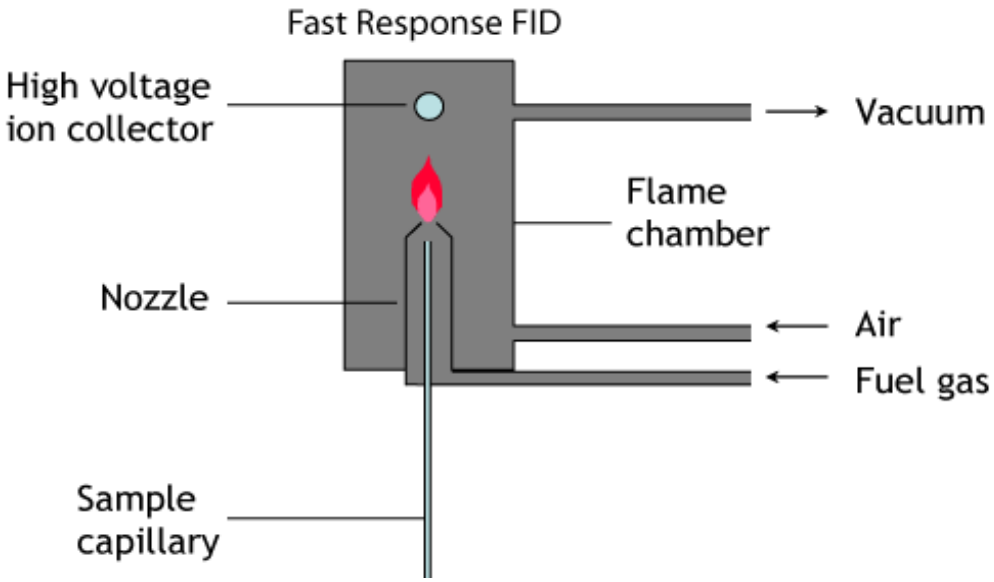


Figure 3.3. Schematic representation of flame ionisation detector (Smith *et al.*, 2005).

Electron capture detector

The electron capture detector (ECD) is a highly sensitive detector capable of detecting picogram amounts of specific types of compounds. The high selectivity of this detector can be a great advantage in certain applications. Compared with the FID, it has much more limited linear response range, generally less than 2 orders of magnitude. The response can also vary significantly with temperature, pressure and flow rate (Robards *et al.*, 1994). The electron captor detector is particularly sensitive to halogen-containing molecules, conjugated carbonyls, nitriles, nitro compounds, and organometallic compounds, but relatively insensitive to hydrogen and alcohol. ECD detectors measure the pulse rate needed to maintain the standing current (Lehrle, 1999).

CHAPTER 4: RESEARCH METHODOLOGY

The procedure of this method employed a combination of supported liquid membrane extraction procedure and that of generic SPE protocol. The SPE steps involved the following steps: conditioning, loading of sample (extraction), washing off possible interferences and/ or substances none specifically bound to the MIP and then finally eluting the bound analytes using a solvent which breaks the specific interaction. An additional step was the separation of the MIP particles from the acceptor solution before washing and elution. The experimental involved varying the extraction time, organic acceptor solvent, the amount of MIP particles, the washing and the elution solvents. The MIP used was synthesized in the laboratory. The experimental further included testing the influence of humic substances to mimic real environment and extraction of plant extracts. Water samples and other plant extracts were extracted too. Finally, validation experiments that included the use of certified reference materials were conducted.

4.1 CHEMICALS

Triazine herbicides (simazine, atrazine, propazine), 17 β-estradiol (E2), methacrylic acid (MAA), 1,1 Azobis(cyclohexane-carbonitrile) (ACCN), 2,2′-Azobis(2-isobutyronitrile) (AIBN), and trimethylolpropane trimethacrylate (TRIM) were purchased from Sigma Aldrich (Darmstadt, Germany). Triazines certified reference material (CRM), TP619 was purchased from (Chester, United Kingdom). The certified reference material consisted of the following triazines dissolved in methanol:

ametryn, prometon, prometryn, propazine, simetryn, simazine and terbutryn. The concentration of CRM was 5 mg l^{-1} of each triazine. Organic solvents were from BDH, (London, England). All solvents used were of analytical grade. Silicone oil was bought from Kynethan Business Management (KBM) (Johannesburg, South Africa). The oil was added as a bath to heat the reaction mixture in the synthesis of molecular imprinting polymer for the compounds.

4.2 EQUIPMENT

4.2.1 Membrane extraction unit

A stainless steel extraction unit was constructed by CE Engineering cc (Johannesburg, South Africa). It consisted of a donor compartment with a volume capacity of 23 cm^3 and an acceptor compartment of 6 cm^3. The upper compartment (acceptor compartment) has a hole on top, see Figure 4.1.

4.2.1 Ultrasonic bath

The 460 Elma Ultrasonic from Braun, (German) was used. The ultrasonic bath was used for dissolution of standards and for the elimination of bubbles from freshly prepared HPLC mobile phase. It was also used to during the extraction of organic compounds from fruit samples.

4.2.2 Centrifuge and crusher

Centrifuge was used to sediment the MIP particles during washing off the template after synthesis. An MSE, Mistral 1000 bench top centrifuge (Hettich, German) was used. A volume (5 ml) of the washing solution was added to the MIP solids and centrifuged at 600 rpm for 30 minutes. This was repeated for 3 times until the entire atrazine and E2 template was completely removed. A Fritsch pulveriser (Germany) was used to crush the apple and lettuce vegetable prior to extraction with methanol. Crushing was achieved at 400 rpm for 60 minutes and repeated twice.

4.3 HPLC CONDITIONS

An HPLC from SRI (Model 210D, LA, California, USA) was used. It consisted of a variable UV detector at which 220 nm was used for the determination of the triazine herbicides and for E2. The equipment was equipped with a rheodyne injection valve with a 20 µl sample loop for injection. The flow rate was set at 1.0 ml/min with an operating pressure in range of 1400 to 1700 psi.

Separation was achieved by a Supelco column (25 mm x 4 mm, 5 µm) (Darmstadt, Germany). Determination was achieved by peak simple chromatographic software (version 3.29) and quantification was done using an external calibration curve.

4.4 PREPARATION OF SOLUTIONS

1000 mg l^{-1} stock solution of triazine herbicides and E2 was prepared in a 50 ml volumetric flask separately. 50 mg of each triazine was weighed and transferred quantitatively into a 50 ml volumetric flask and dissolved in methanol. The volume was made to the mark using methanol. From the 1000 mg l^{-1}, a 10 mg l^{-1} stock solution was prepared which was used for spiking low concentrations in the region of 1 µg l^{-1} level. Standard solutions ranging from 0.1 mg l^{-1} to 2 mg l^{-1} were prepared and used for the calibration curve. 1000 mg l^{-1} stock solution of E2 was prepared by dissolving 50 mg of E2 in acetonitrile. The volume was diluted to the 50 ml mark with acetonitrile. The calibration curve was diluted from the standards solution ranging from 1 to 7 mg l^{-1}. All stock solutions and standards solutions were stored at 4^{o}C and were prepared every month.

The composition of the mobile phase was 60% deionised water and 40% acetonitrile. The prepared mobile phase was filtered twice through hydrophilic membrane of 0.45 µm pore size using a vacuum pump and then sonicated for 20 minutes in an ultrasonic bath to get rid of the bubbles. The phosphate buffer was used during the extraction of organic compounds from vegetable and fruit extract. The phosphate buffer at pH 7 was prepared by dissolving 20.75 g Na_2HPO_4 and 5.03 g NaH_2HPO_4 in 500 ml deionised water.

4.5 SYNTHESIS OF MOLECULARLY IMPRINTED POLYMERS (MIPs)

The bulk MIP synthesis protocol was taken from Bjarnasson *et al.,* (1999) with slight modification. For the preparation of triazine MIP, 1 mmol of atrazine, 8 mmol of monomer (MAA), 25 mmol of cross-linking agent (TRIM) and 50 mg of initiator (ACCN) were dissolved in 7.5 ml of toluene in an air-tight glass vial. For the E2 MIP, imprint molecule 17 β-estadiol (0.145 mmol), functional monomer MAA (0.696 mmol), cross linker, TRIM (0.296 mmol) and 50 mg of initiator (AIBN) were dissolved in 8 ml of acetonitrile. Each polymerization mixture was sonicated for 5 minutes in round bottom flasks. The mixture was then purged with nitrogen for 5 min to remove oxygen. The polymerisation was done overnight at 60°C in an oil-bath. The bulk polymers were successively ground in a mortar and crushed with ceramic beads. Particle sizes in range of 25-90 µm were then collected by sieving under water using two stainless steel sieves.

As indicated earlier, one of the problems with the usage of MIP is the inability to remove the entire template (Cacho *et al.,* 2004). To completely remove the template, the MIP was exposed to Soxhlet extraction using methanol: acetic acid (1:1) for 12 hrs. After the Soxhlet extraction, the polymers were centrifuged in 3 ml of methanol: water (9:1) for 30 min at 600 rpm. Three rounds of such centrifugation and decanting was adequate to completely remove the atrazine template when analysed by an HPLC-UV. The polymers were then dried overnight. Blank imprinted polymers were prepared and treated in the same, except the print molecule was excluded in the polymerisation mixture.

4.6 EXTRACTION PROCEDURE WITH MMLLE-MIP TECHNIQUE

Preparation for the extraction involved first filling 23 ml of the sample in the lower compartment (Figure 4.1). The membrane previously soaked for 5 minutes in organic liquid (toluene for triazines and hexane – ethyl acetate for E2) at optimized conditions was placed on top. The upper compartment was then screwed. 2.5 ml of the organic liquid followed by 10 mg (for triazines) of MIP beads were then added. The top hole was then closed and the unit was ready for extraction after shaking the unit to disperse the added MIP beads. For the extraction of E2, 2.5 ml of hexane-ethyl acetate (3:1) mixture and 30 mg of MIP was used.

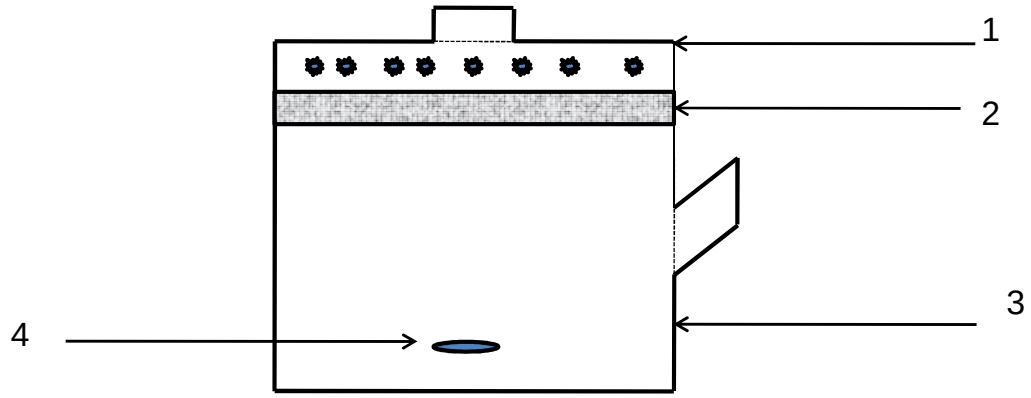


Figure 4.1: Schematic set-up of the extraction unit. 1=Acceptor compartment filled with 2.5 ml of the acceptor solution and the MIP (represented as black dots) (10 mg for triazine extraction and 30 mg for E2 extraction), 2 = PTFE membrane soaked with acceptor solution, 3 = Donor compartment with filled with 23 ml of aqueous sample, 4 = magnetic stirrer.

Triazine compounds were extracted from aqueous phase into organic acceptor phase due to their solubility where they were re-extracted onto MIP particles. After 90 minutes of extraction, MIP particles were separated from the organic phase by passing the whole content through a 0.1 µm PTFE syringe filter and the MIP beads were trapped. Non specific bound compounds were washed off the MIP by passing through 2 ml of dichloromethane. Using a small syringe, 3 ml of methanol in water (90:10%) was passed through to elute the specifically bound triazines which was then analysed on HPLC.

The same protocol was used for the E2 extraction. The differences being the extraction time which was 60 minutes and washing off non - specific bound analytes was achieved by passing 2 ml of the fresh acceptor solution (hexane – ethyl acetate (3:1). The extraction process flow diagram is shown in Figure 4.2.

In some experiments, MMLLE was performed without the MIP in the acceptor phase so as to compare the selectivity to the combination. For this purpose, after MMLLE, the acceptor solution was taken out and left to evaporate at room temperature. Then 1 ml of methanol was added and injected into the HPLC. This step was performed so as the sample medium was changed from toluene to methanol to be injected into a reverse phase column.

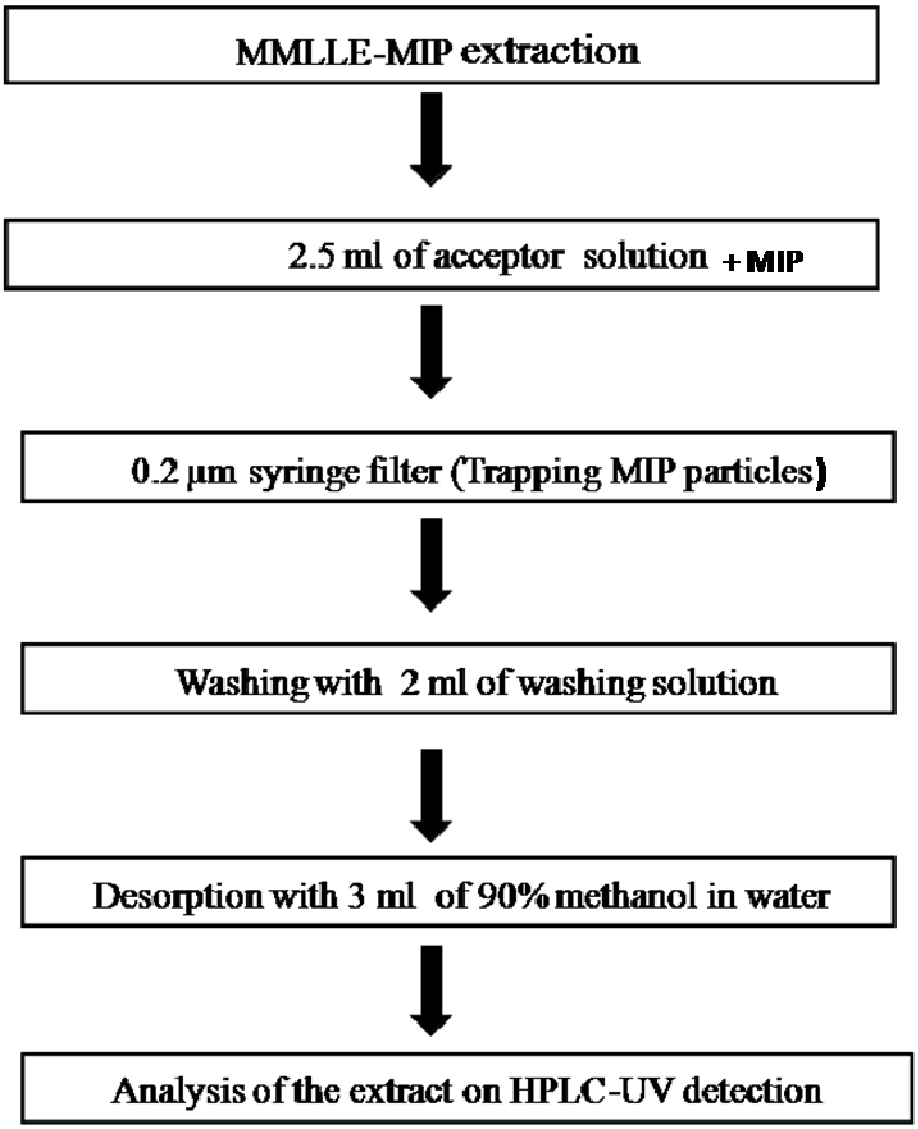


Figure 4.2: Summary of the extraction sequence. After MMLLE-MIP extraction, the acceptor phase was passed through a 0.2 µm syringe filter on which the MIP beads were retained. MIP beads were washed with 2 ml washing solution (dichloromethane for triazines and hexane – ethyl acetate for E2) followed by desorption with 3 ml 10% water in methanol.

4.7 OPTIMISATION EXPERIMENTS

Since the extraction in MMLLE-MIP is based on the mass transfer of the analytes from the aqueous sample to the organic solvent in the acceptor and finally on to the cavities on the MIP particles, several parameters need to be optimized. Such parameters as the type of organic solvent, the amount of MIP and extraction time have a direct influence to the amount of target analyte bound to the MIP.

As in solid phase extraction, after the loading or percolation stage, the MIP need to be washed with a moderately polar solvent that removes non specific bound compounds on the surfaces of the MIP. To achieve good selectivity and enrichments, a good washing and elution solvent will have to be carefully selected. The size of the unit was chosen in the previous work (Nemulenzi *et al.*, 2008). It was found out the smaller unit produced better extraction efficiencies because of improved mass transfer. Nemulenzi *et al.*, (2008) optimized the washing, desorption solvent and extraction time for E2 extraction. The optimum extraction time chosen for E2 extraction was 60 minutes and 90% methanol in water was chosen as the best eluting solvent. The following parameters for E2 extraction were either improved or optimized in this study namely; amount of MIP mass, the type of organic acceptor and ultrasound assisted after binding onto MIP particles.

4.7.1 Varying the amount of MIP in the acceptor phase

Increasing the amount of MIP increases the amounts of binding sites. Different amounts of MIP particles of 5, 10, 15 and 20 mg were added into the acceptor phase

so as to study how it influences the extraction process of triazine compounds. Extraction time of 60 minutes and hexane-ethyl acetate (3:2) was used as organic liquid. Deionised water containing 1 mg l^{-1} mixture of triazine compounds was used as aqueous sample. 3 ml methanol was used as desorption solvent after washing with 2 ml of the acceptor solvent.

In E2 extraction, 7, 15, 30 and 50 mg of MIP were varied in the acceptor phase so as to study how the different mass influences the extraction process of E2. An extraction time was 60 minutes and hexane-ethyl acetate as organic liquid.

4.7.2 Varying acceptor solution

MIPs are known to exhibit better selectivity when the extraction is performed in the organic medium and in particular the organic solvent used as porogen. Carabias-Martinez *et al.,* (2006) extracted triazines herbicides and their metabolites directly from aqueous solution. The extraction of the triazines was due to non - specific interaction rather than selective recognition. In this set-up, triazine compounds have to be extracted onto MIP particles in the organic solvent. The first extraction involved supported liquid membrane extraction. It is therefore important that the organic liquid used has high partition coefficient for the analytes. Secondly the organic liquid should not interfere with analyte binding onto MIP particles therefore moderate polar and aprotic solvents are preferred that do not interfere with analyte binding onto MIP.

To study the influence of organic acceptor solvent on the extraction process for triazines; acetonitrile, hexane, toluene and different combinations of hexane-ethyl

acetate were studied. The volume of the acceptor solution was maintained at 2.5 ml. Extraction time of 60 minutes and 7 mg of MIP were used. Deionised water containing 1 mg l^{-1} mixture of triazine compounds was used as aqueous sample. 3 ml methanol was used as desorption solvent after washing with 2 ml of fresh acceptor solvent. The experiments were done in duplicates. The physical properties of organic solvents examined are shown in table 4.1 below.

Table 4.1: Properties of the organic solvents studied to determine the best organic acceptor (http://organicdivision.org). 2.5 ml of each acceptor was used as the acceptor solution.

Organic Acceptor	Boiling Point (^oC)	Dielectric strength	Solubility in water (g/100g)	Relative polarity	$logK_{ow}$
Acetonitrile	81	37	Miscible	0.460	-1.3
Ethy acetate	77	6	8.7	0.228	0.73
Hexane	69	1.89	0.014	0.009	3.9
Toluene	111	2.38	0.05	0.099	2.69

The influence of the organic acceptor for the E2 extraction was studied through liquid-liquid extraction technique. 20 ml of toluene, diethyl ether, cyclohexane, hexane and hexane-ethyl acetate (3:2) solvents were mixed each with 100 ml of deionised water spiked with 1 mg l^{-1} of E2 in 25 ml separating funnel. The funnels were vigorously mixed for about 30 minutes and then left to stand. When the phases

were separated, the aqueous phase was separated and E2 concentration was determined.

4.7.3 Varying the washing and desorption solvents

One important step in SPE is the washing of the sorbent following sample loading. This is necessary to remove analyte interferences prior to analyte elution. Different washing solvents were not evaluated in detail but solvents such as ethyl acetate, acetonitrile, butyl methyl ketone, toluene and dichloromethane were screened to see the increase in amount extracted after desorption. Extraction time was 60 minutes and 10 mg of MIP was used. Deionised water containing 1 mg l^{-1} mixture of triazine compounds was used as aqueous sample. 2.5 ml toluene was used as acceptor with 3 ml methanol as desorption solvent after washing with 2 ml of dichloromethane. The experiments were done in duplicates.

In order to desorb all the amount of triazines, acetonitrile, methanol, water and 90 % methanol in water were tested. The physical properties of the studied desorption solvents are shown in Table 4.2. Extraction time was 60 minutes and 0.1 mg l^{-1} of triazine mixture spiked in deionised water was extracted with 10 mg of MIP in the organic acceptor solution (toluene). 2 ml of dichloromethane was used as a washing solution of MIP followed by desorption of the solvents.

Table 4.2: Properties of the elution solvents studied. In other cases a mixture of two elution solvents was also studied (http://organicdivision.org).

Elution solvent	Boiling Point (^{o}C)	Dielectric strength	Hydrogen bond donor propensities	Hydrogen bond acceptor propensities	Relative polarity
Acetonitrile	81	37.0	0.07	0.32	0.460
Water	100	78.5	1.17	0.47	1.000
Methanol	64.6	1.89	0.43	0.47	0.460

The washing and desorption solvents for E2 extraction were taken from the previous study as mentioned before. Hexane – ethyl acetate (3:2) and methanol – water (90:10) were chosen as the organic acceptor and elution solvent respectively.

4.7.4 Extraction time variation

The mass transfer of the target compounds from the donor compartment into the bulk of organic acceptor phase and the subsequent binding onto MIP particles is time dependant. To study the influence of time on the extraction process, extraction time was varied from 10 to 120 minutes. For these experiments, 2.5 ml of toluene was used as acceptor organic phase with 10 mg of MIP particles. Trapped analytes were desorbed by 3 ml methanol in water (90:10 v/v %) after washing with 2 ml of dichloromethane. The sample was deionised water spiked with 0.1 mg l^{-1} of the triazine mixture.

The extraction was carried for the 10, 30, 60, 90 and 120 minutes. The experiment would be stopped after each extraction time and the donor, organic acceptor (toluene), MIP wash (dichloromethane) and MIP elution (methanol/ water) were then injected into the HPLC for analysis. The donor solution was injected into the HPLC without further treatment. Organic acceptor and dichloromethane samples were evaporated to dryness at room temperature and then re- dissolved in 0.5 ml of reverse – phase HPLC compatible mobile phase solvent which in this case was methanol - water.

The methanol - water elution solvent was evaporated from 3 ml to 0.5 ml at the same conditions described earlier. This was done in order to increase the enrichments factors of the triazines.

For E2 extraction, the extraction time was taken from the previous study (Nemulenzi *et. al.*, 2008) and it was 60 minutes.

4.7.5 The effect of donor concentration

During real sample extraction, different concentrations of triazines are encountered. Therefore it is important to study how the triazines donor concentration may affect the extraction efficiencies and enrichments factors. To determine if the extraction efficiency is dependent on the concentration in the donor phase, varying concentrations (2.5, 5, 10 and 30 $\mu g\ l^{-1}$) were extracted at optimum conditions of the MMLLE – MIP technique. For E2 extraction, only donor concentration of 0.1 and 0.5 $mg\ l^{-1}$ were varied due to low detection limit.

4.7.6 The effect of humic substances on the extraction efficiency

The effect of humic substances on the extraction efficiency of triazines was studied. This is because humic substances are frequently found in natural waters. There are normally found at concentrations around 5 mg l^{-1} (Zoritha *et al.,* 2008). In solid phase extraction, it has been noted to give a huge peak at the beginning of the chromatogram as it is also extracted and co-eluted by the most common non-selective sorbents (Shen *et al.,* 1994, Megersa *et al.,* 1999).

To determine the effect of humic substances on the extraction of triazines and on the selectivity of the technique, humic substances were added to deionised water. The preparation of humic substances solution was adapted from Bjarnasson *et al.,* (1999). Humic substances solution was prepared by grinding an appropriate amount of humic substances in a mortar and mixed with deionised water to give concentrations ranging from 0 to 20 mg l^{-1}. The mixture was stirred for a week and then filtered through 0.45µm filters.

The filtered humic substances solutions were spiked to a total final concentration of 30 µg l^{-1} triazines. The concentration of humic substances solutions extracted was 0, 5, 10 and 20 mg l^{-1} containing triazines mixtures. Extraction with MMLLE – MIP technique was performed at optimised conditions.

4.7.7 The effect of ultrasound assisted binding on E2 binding

It is important that all the target analytes that has been extracted into the organic phase should bind onto MIP beads. E2 showed slow binding on to the MIP. In order to increase the re-binding of the E2, ultra sound assisted re-binding of E2 onto MIP beads were investigated. For these experiments, after 60 minutes extraction with LM-MIP unit, the whole acceptor phase was transferred into vial and sealed. The samples were then subjected to ultra sound for 5, 10, 15 and 30 minutes. The probe depth in the sample solution was 1cm.

4.8 MMLLE - MIP TECHNIQUE EXTRACTION OF FOOD SAMPLES

4.8.1 Food samples

To demonstrate the potential of the extraction technique to food samples, apple fruit and lettuce vegetable were investigated. Both food samples were bought from a local food shop and were picked randomly. The sample preparation technique was adapted from Bjarnasson *et al.,* (1999) with slight modification. Food samples were crushed and homogenised and 4 grams of vegetable and 40 grams of the apple were taken. In each of these, 160 ml of methanol was added. The samples were then sonicated for 30 minutes and left to stand for an hour. Then it was filtered through 0.45 μm filter paper. The filtrate was left to evaporate overnight. To the thick liquid that remained, 150 ml of phosphate buffer at pH 7 was added. This was filtered once more and filtrate was spiked with appropriate amount of triazines and then extracted with MMLLE – MIP extraction technique. To compare selectivity of the MIP to NIP,

extraction was performed with NIP in the acceptor phase instead of the MIP. The NIP was washed and eluted as described for the MIP. The selectivity of the extracts after MIP elution in MMLLE – MIP combination to that remaining in the bulk organic acceptor was also compared. In this case solvent exchange was made as to allow for injection of organic acceptor into the RP – HPLC as described in section 4.7.4.

4.8.2 Wastewater samples

To demonstrate the potential of the extraction technique to real samples for the E2 study, spiked wastewater from a local treatment plant was extracted. Wastewater influent and effluent was collected from one of the wastewater treatment plants used to treat both industrial and household waste west of Johannesburg. The pH of the wastewater was measured and the water was filtered through 0.45 μm filter paper. Wastewater was spiked with concentrations of 0.1 and 0.5 mg l^{-1} E2. Each spiked sample was extracted in the same way as described in section 4.6. Each experiment was repeated twice.

4.9 VALIDATION OF THE DEVELOPED TECHNIQUE

In order to establish the validity of the developed MMLLE – MIP technique, it was applied to the extraction of triazines in a certified reference material (TR619). Samples of wastewater and deionised water spiked with 5 μg l^{-1} reference material were extracted in triplicates at optimized conditions. Extraction efficiency, selectivity and concentrations obtained were compared to those determined when non- standard

solutions were extracted and to the originally spiked amounts for the reference materials.

4.10 ENRICHMENT FACTOR AND EXTRACTION EFFICIENCY

The enrichment factor, E_n, is a ratio of concentration (C_A) obtained in the acceptor phase or after desorption from the MIP and /or after subsequent solvent reduction to that in the original donor sample (C_I) (Jonsson *et al.,* 2001, Chimuka *et al.,* 1998).

The enrichment factor is important in that it allows the calculation of the detection limit of the MMLLE – MIP technique. The enrichment factor was calculated as follows.

$$E_n = C_A/C_I \qquad (4.\ 1)$$

The extraction efficiency (Jöhnsson *et al.,* 2001), E_n, is defined as the fraction of analyte extracted from the donor phase into the acceptor phase or after desorption from MIP and was calculated from:

$$E_A = n_A/n_I \qquad (4.\ 2)$$

or

$$E_A = c_A v_A / c_I v_1 \qquad (4.\ 3)$$

or

$$E_D = 1 - n_F/n_I \quad (4.4)$$

Where: n_A and n_I are the total amounts of analyte from the collected acceptor and the extracted sample solutions determined by a calibration curve, respectively. n_F is the amount not extracted into the membrane in the sample according to our present set-up. c_A and c_I are the concentrations of the analyte in collected acceptor and in the extracted sample. v_A and v_I are the volumes of the acceptor and the sample. If all analytes that were extracted into the membrane are collected into the acceptor phase, $E_A = E_D$. In most applications of liquid membrane extraction techniques, E_A is measured. The extraction efficiency and enrichment factor are both constant at specified extraction conditions such as extraction time, amount of MIP, desorption solvent, type of organic acceptor solvent, stirring speed etc (David and Sandra, 2007). The extraction efficiency is a measure of the mass transfer kinetics in the MMLLE techniques. High extraction efficiency represents fast mass transfer and vice versa. In this set-up, this can be divided into two parts, diffusion into the bulk acceptor solution from the donor compartments and binding onto MIP particles.

4.11 PERMEATION STUDIES

In permeation studies, the movement of analytes from the donor compartment into the bulk acceptor was assumed to follow first order reactions according to equation 4.5:

$$r = -d[A]/dt = k[A] \quad 4.5$$

Where A is the concentration of the triazine in donor compartment. k is the first order rate constant which has units of 1/time. Equation 4.5 above when integrated gives:

$$\ln[A]_t = - kt + \ln[A]_o \qquad 4.6$$

Where $[A]_o$ and $[A]_t$ are the final and the initial concentration respectively in this case, in the donor compartment after extraction time, t. A plot of $\ln\{[A]_t/[A]_o\}$ against time gives a straight line with a slope –k. The half life ($t_{1/2}$) is independent of the starting concentration and is given by:

$$t_{½} = 0.693/k \qquad 4.7$$

In order to calculate k and $t_{1/2}$ in MMLLE – MIP technique, the concentration remaining in the donor compartment was measured after time t (see section 4.7.4 for experimental details). These experiments were done with or without MIP in the acceptor phase so as to evaluate the influence of the MIP on k and $t_{1/2}$.

4.12 QUANTIFICATION AND QUALITY ASSUARANCE

Quantification of the extracts for triazines herbicides were performed by external calibration curve that was linear in concentration range of 0.1 to 2.5 mg l^{-1}. Typical standard chromatogram and calibration curve for triazine study are shown in Figures 4.3 and 4.4 below.

A number of steps were taken to ensure quality of the results obtained in any experimental part. This included repeating experiments two or more times. Certified reference standards as discussed earlier were also used as part of quality assurances.

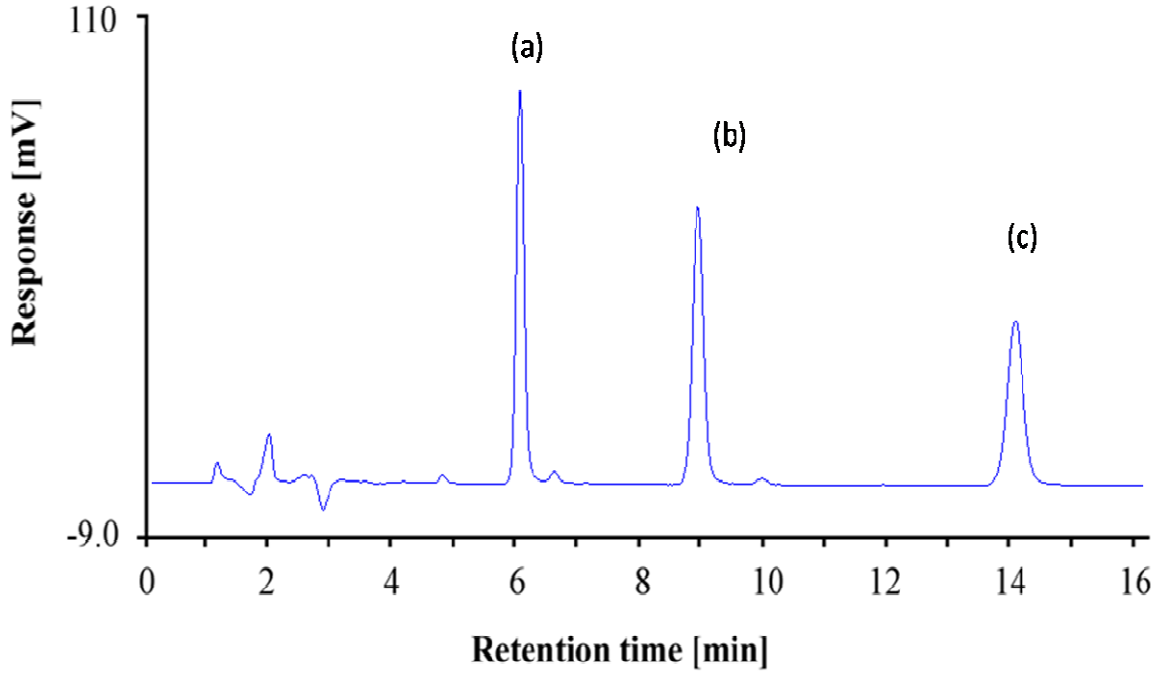


Figure 4.3: A typical chromatogram of a 1 mg l^{-1} triazines standard injection. Where (a) = simazine, (b) = atrazine and (c) = propazine

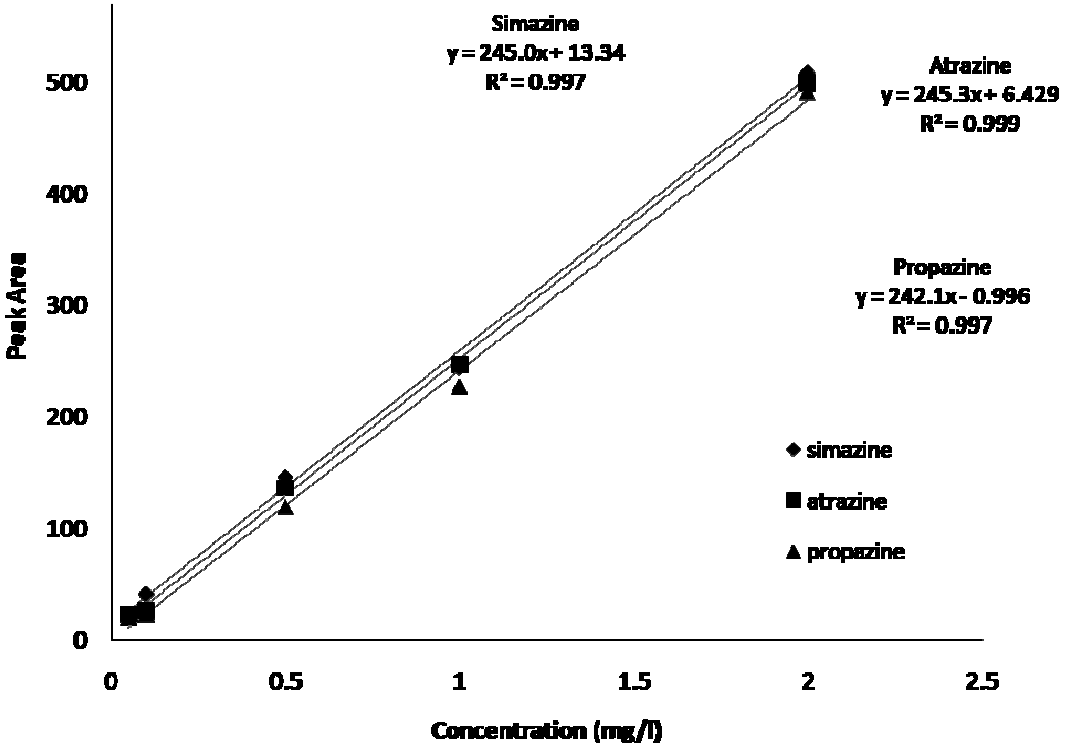


Figure 4.4: A typical calibration curve of triazines.

CHAPTER 5: RESULTS AND DISCUSSIONS

The aim of the work was to develop a technique for the selective extraction and pre-concentration of organic chemicals from complex aqueous samples. Triazines herbicides and 17 β-estradiol were extracted from plant extracts and wastewater respectively and quantified by an HPLC-UV detection. The technique showed molecular recognition and sample clean-up of these chemicals from complex matrices. Results obtained includes the preparation of the MIP particles, optimisation of the method, testing its validity and its application to spiked samples.

5.1 SYNTHESIS OF MIPs

MIP preparation for triazine compounds is well documented in literature (Turiel *et al.,* 2007, Sambe *et al.,* 2007, Chapuis *et al.,* 2003). Although nowadays MIPs are often synthesised in the form of spherical particles, thus avoiding grinding and sieving, in this application crushed monoliths were suitable. This option was chosen during the synthesis of bulk MIPs, monomer concentrations are higher than for example in precipitation polymerisation, resulting in higher yields in binding sites. However the MIPs particles for E2 were prepared by both bulk and precipitation techniques. The precipitation technique was employed in the previous study by Nemulenzi *et al.,* (2008). The morphologies and porosities of the resulting E2 imprinted materials were characterized by scanning electron microscopy using JSP at 2000x magnification. Figure 5.1 shows that from bulk polymerisation, bigger particles were prepared compared to precipitation polymerisation. In the latter

technique smaller particles were formed and that formed clusters or aggregates. The average particle diameter in bulk polymerization was about 20 µm while in precipitation polymerization was about 1 µm.

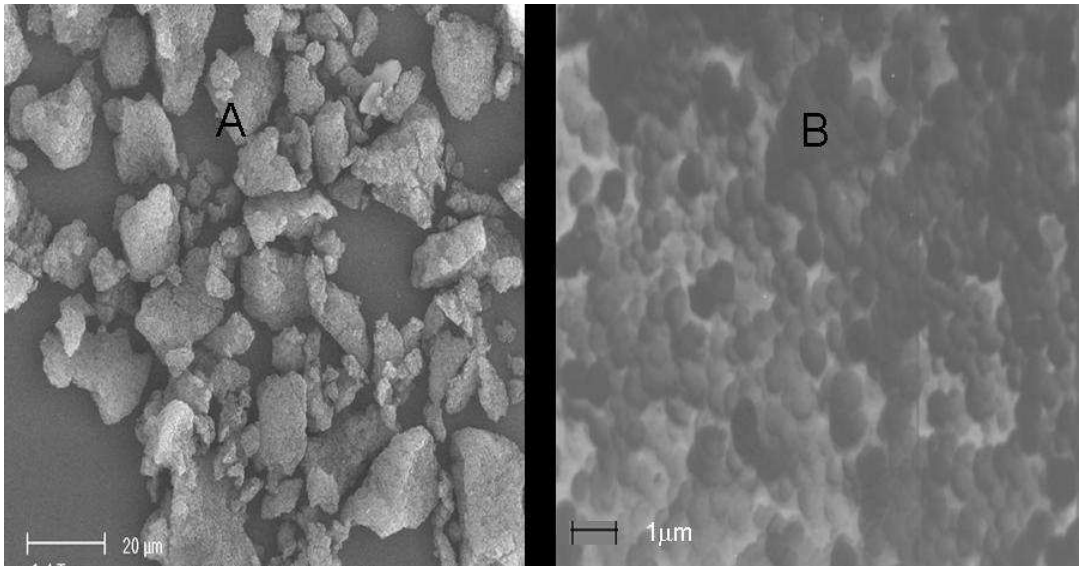


Figure 5.1 (a) represents the morphology of the MIPs prepared using bulk method and (b) represent the morphology of the MIPs prepared by precipitation method (Nemulenzi *et al.,* 2008).

The MIP particles obtained by bulk polymerisation shown in Figure 5.1 (a) are irregular with a mixture of big and smaller particles. Ye *et al,* (2001) noted that bigger MIP particles lead to slow diffusion of analytes in and out of the pores and hence poor access to binding sites. In the previous study by Nemulenzi *et al.,*(2008), MIP particles prepared by bulk and precipitation polymerisation were compared as part of the acceptor solution in the extraction of 17 β – estradiol. Much better extraction was obtained from MIP particles prepared by precipitation because of

increased surface area. For the triazine herbicides extraction, only bulk polymerisation technique was chosen and employed because bigger MIP particle were easily retained by the 0.20 µm syringe filter during the separation of the MIP particles from the bulk organic acceptor solution.

Complete template extraction from the prepared MIP is a crucial step as this creates selective cavities ready for analyte recognition and prevents breeding during application. Several procedures have been reported in the purification of MIPs ranging from simple repeated washing with a mixture of organic solvents (usually methanol, acetonitrile or acetic acid) (Turiel *et al.,* 2007, Sambe *et al.,* 2007, Chapuis *et al.,* 2003), Soxhlet extraction (Tuna *et al.,* 2006; Koohpaei *et al.,* 2008), microwave assisted extraction (Bravo *et al.,* 2005) and accelerated solvent extraction (ASE) (Ju *et al.,* 2007). Ju *et al.,* (2007) used 300 ml of methanol-acetic acid (90:10, v/v) at 80^{o}C for the extraction of nicotine template from the MIP synthesized by noncovalent protocol. The template removal efficiency was 94.2%. Koohpaei *et al.,* (2008) reportedly used Soxhlet extraction for the removal of ametryn template from the polymer. The template was removed using a two-step procedure (methanol: acetic acid, 9:1 for 18 hours as a first step and methanol for 6 hours as a second step).

In this study, Soxhlet extraction for 12 hours using methanol: acetic acid (1:1) was used. After the Soxhlet extraction, the polymers were centrifuged in 3 ml of methanol: water (9:1) for 30 min at 600 rpm. The final decanted solution was pre-concentrated to 0.5 ml and injected into the HPLC to test for impurities. No template peak was detected. This suggests that most of the template was removed from the

polymer. Figure 5.2 shows the chromatogram of the final decanted solution during the template removal from the triazine MIP.

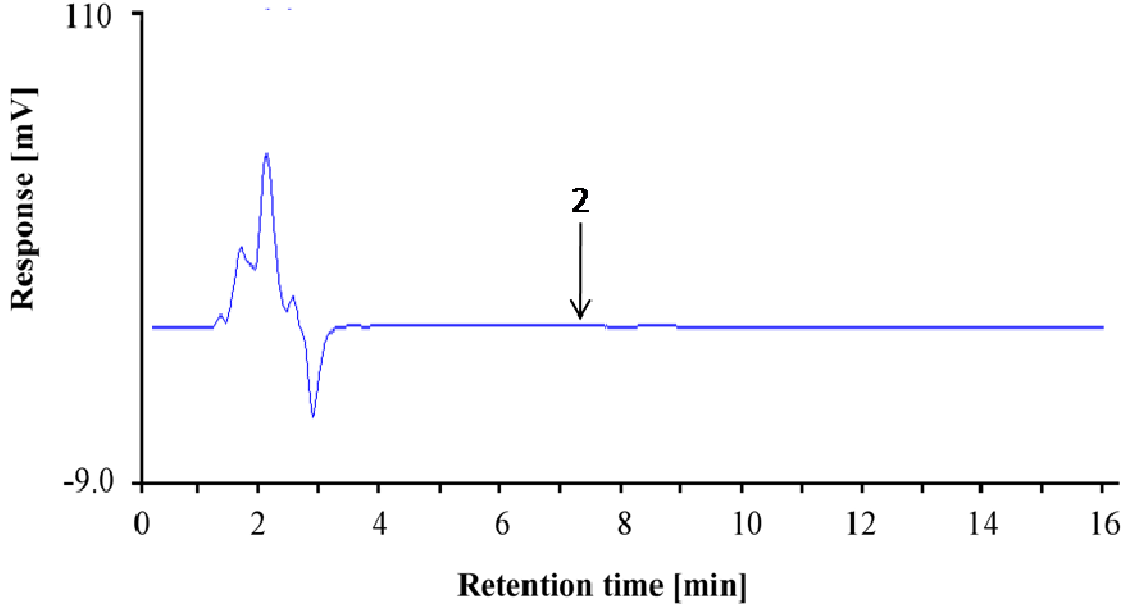


Figure 5.2 The concentrated final decanted triazine MIP wash. The clean chromatogram indicates the complete removal of the template (atrazine). 2 represents where atrazine peak (the template) is expected.

5.2 OPIMISATION RESULTS

5.2.1 Variation of the amount of MIPs in the acceptor phase

Several factors govern the binding capacity of MIP particles. These factors include number of binding sites, surface area, the pore size and the pore size distribution. The amount of MIP in the acceptor phase was varied as described in the experimental. As

expected, an increase in amount of MIP particles lead to an increase in the target analytes bound (Figure 5.3).

The MIP prepared with atrazine as template for the triazine herbicides extraction showed cross- reactivity as other herbicides were also extracted. As expected atrazine was bound more efficiently compared to the other triazines. However, the structure of propazine is close to that of atrazine and thus both compounds are bound similarly well. When comparing the amount of E2 bound on the bulk MIP to the amount of E2 bound on the precipitation MIP, it was shown that the latter was better. The amount of E2 bound to the later was approximately double than that of the former (Nemulenzi *et. al* 2008). The low surface area of the MIP prepared by bulk polymerization is the main cause of these low extractions.

Apart from having low surface area, several researchers have reported that the process of crushing and sieving the bulk polymer after polymerisation can break the imprinted sites and shrinkage of cavities may occur after removal of template with polar organic solvents (Turiel, 2001). Some of these factors may have influenced MIP binding.

It should be noted here that the amount of MIP used is much less compared to that in SPE. In SPE, using a cartridge, an amount of sorbent close to 200 mg is packed (Hennion, 1999). In our set – up it is generally not necessary to have higher amount of MIP sorbent as in SPE since extraction is not directly onto the MIP. Matrix components that compete for the same binding sites are excluded by the liquid

membrane. This therefore allows using lower amounts of sorbent compared to SPE technique. This also explains why the proposed MMLLE – MIP technique is supposed to give cleaner extracts.

E2 showed slow binding kinetics. The concentration of the E2 remaining in the bulk acceptor phase was determined together with the amount of E2 bound to the MIP and the ratio was found to be 1.5 to 1. Even if the amount of MIP was increased the ratio remained almost constant. This does indicate the slow binding kinetics of E2 onto the MIP particles hence higher amounts of MIPs particles (30 mg) were used compared to the amounts used in herbicides extraction (10 mg).

The triazine herbicides showed better binding kinetics and lesser amounts of MIPs were studied. The amount of MIP in the acceptor phase may still be increased beyond 20 mg for the herbicides extraction which may improve the extraction efficiencies. However there is an upper limit beyond which the MIP will affect the mass transfer from the membrane into bulk acceptor solutions. This is due to the fact that too much MIP can block the inside surface of the membrane, thus slowing diffusion of the compounds into the bulk acceptor solution. The other problem with increasing the amount of MIP is that too much MIP particles would clog the membrane filter during separation of the MIP from the organic acceptor hence washing and elution solvents may not pass through the MIP.

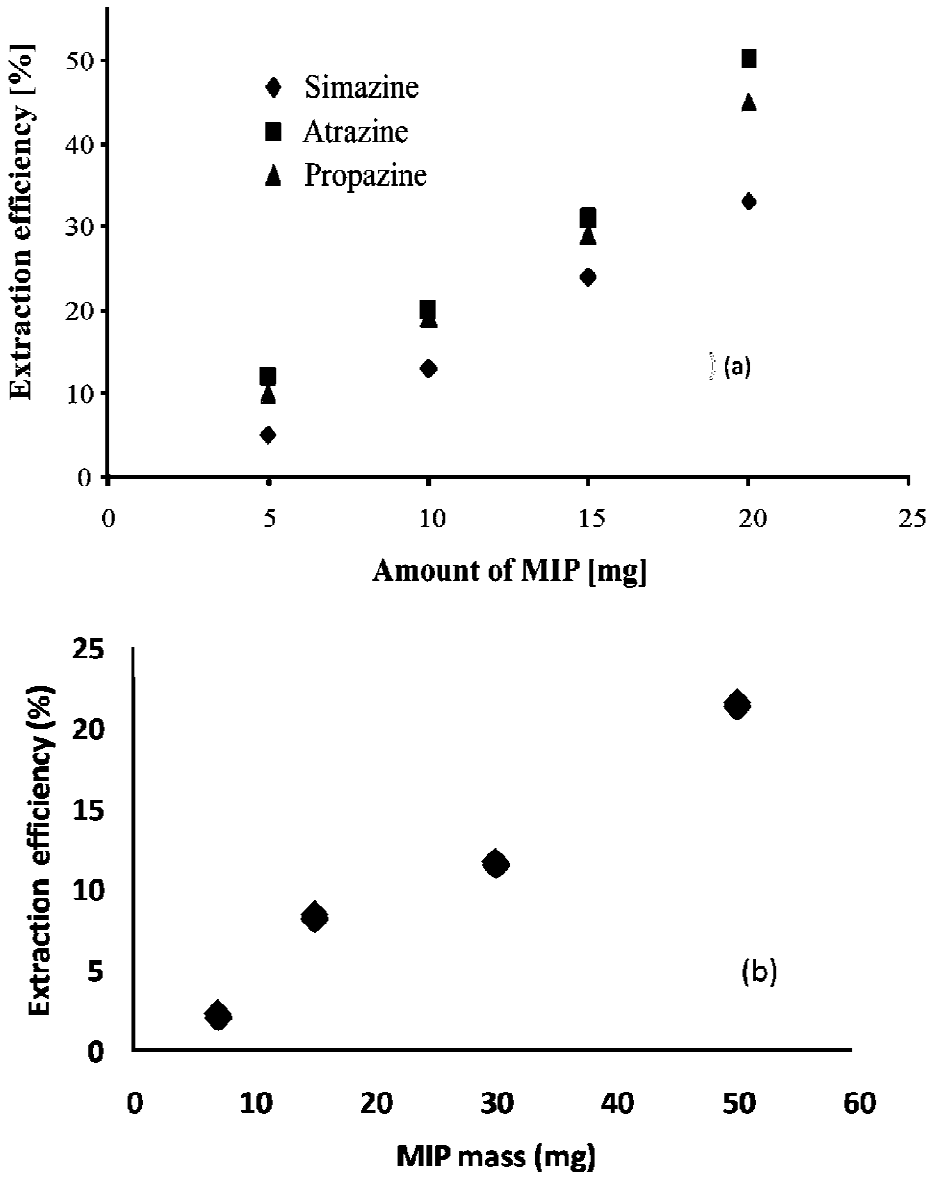


Figure 5.3: The effect of varying the amount of MIP in the organic acceptor solution (hexane –ethyl acetate; 3:2). (a) 1 mg l^{-1} of triazine mixture spiked in deionised water as sample. (b) 1 mg l^{-1} of E2 spiked in deionised water was extracted as a sample. Extraction time was for 60 minutes and fresh acceptor solution (2 ml) was used as a washing solution of the MIP followed by 3 ml of methanol as desorption solvent.

5.2.2 Influence of the organic acceptor on the binding of triazines onto MIP particles

From other studies (Ferrer *et al.*, 2000, Chapius *et al.,* 2003) it has largely been demonstrated that MIPs offer the highest selectivity when extraction is performed in the solvent used for their preparation. In this set-up, the best solvent is the one that gives high partitioning of the target analytes from the aqueous phase and at the same time allows for their strong binding to the MIP particles. Factors that govern analyte-solvent interaction are van der Waals forces that include hydrogen bonding, dipole-dipole interactions and London dispersion forces. Less solvating media would be preferred for target analyte binding onto MIP particles, whereas good solvation is needed for high partitioning of triazines and E2 from the aqueous phase into the organic solvent. Several solvents (toluene, hexane, hexane- ethyl acetate (3:2) and hexane-ethyl acetate (3:1)) were screened as possible acceptor solution in order to achieve an optimal balance between the two effects.

One would expect an organic acceptor which is apolar, which is hexane in this case, to give higher extraction efficiencies compared to the other solvents (Table 4.1). But from the results shown in Figure 5.4 indicate that more triazines were extracted with toluene. This might be because that toluene as the porogen solvent in polymerization, could influence the degree of polymer chain solvation and adjusted the solvation of microenvironment of the binding sites similar with in the developing polymer (Spivak, *et al.,* 1997). Triazine compounds have similar aromatic ring with toluene. Resulting interaction between triazines and toluene is therefore expected to be high.

The addition of ethyl acetate to hexane resulted in a negative influence. This could be attributed to carbonyl groups that could compete for the binding sites due to hydrogen bonding to the monomer in the polymer. Acetonitrile gave the least amount extracted not included in the figure. This could due to the fact that it is water soluble and could not make a stable liquid membrane. Atrazine has the highest extraction efficiency because the MIP was prepared using atrazine as the template hence it was bound more effectively compared to propazine and simazine.

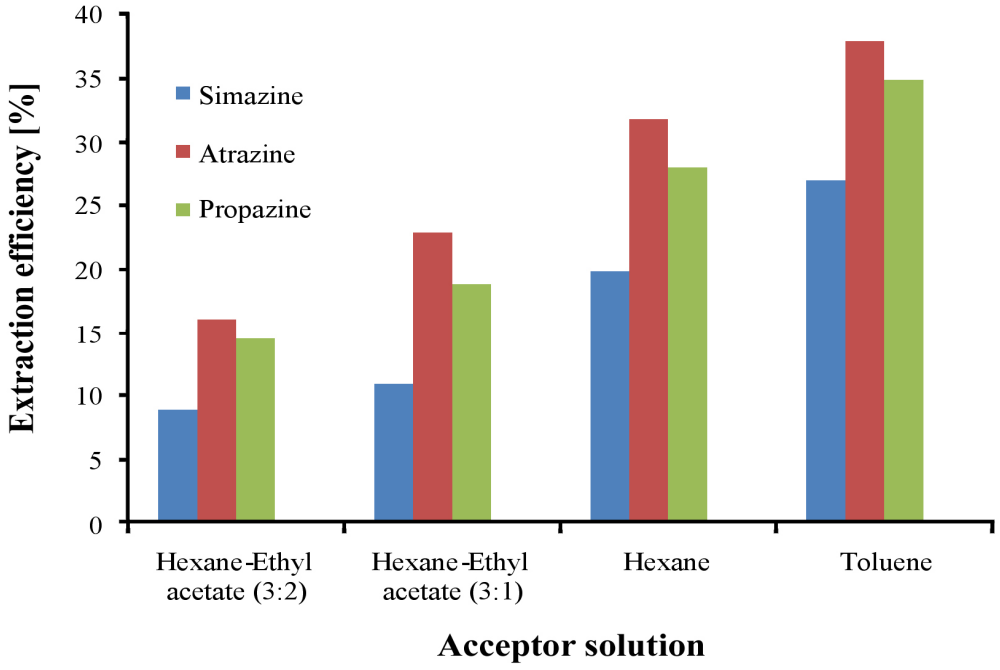


Figure 5.4: Influence of acceptor solution on extraction efficiency. Extraction time was for 60 minutes and 1 mg l^{-1} of triazine mixture spiked in deionised water was extracted with 7 mg of MIP in the organic acceptor solution. 2 ml of hexane – ethyl acetate (3:2) was used as a washing solution of the MIP followed by 3 ml of methanol as desorption solvent.

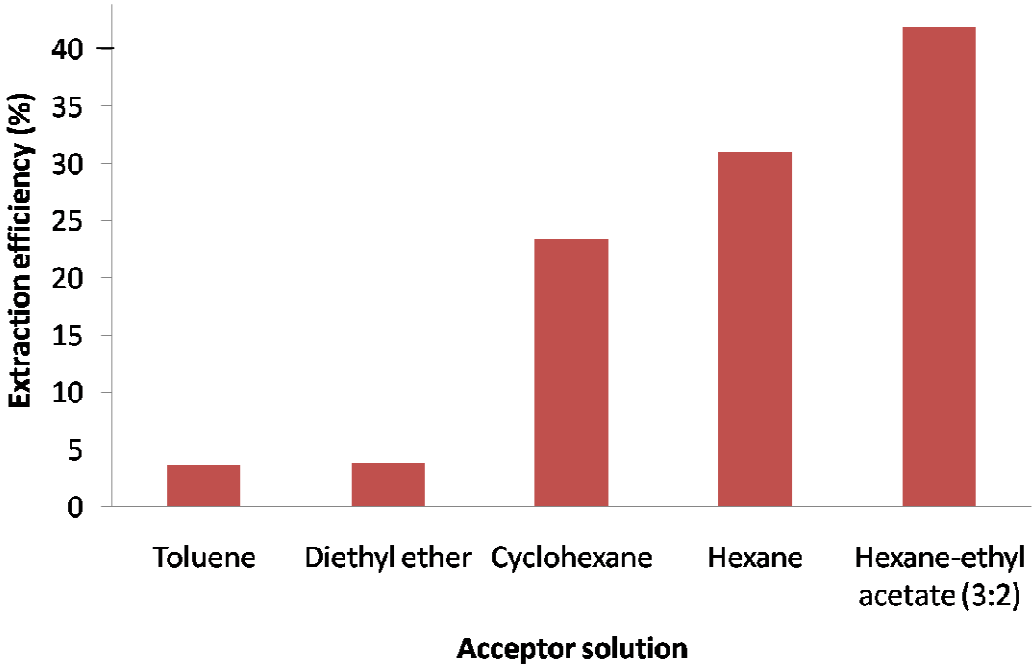


Figure 5.5: Varying organic solvent in liquid-liquid extraction. 100 ml of deionised spiked with 1 mg l^{-1} of E2 was extracted with 20 ml of the organic acceptor.

In E2 optimisation of acceptor solution, several solvents were thus screened by comparing the amount of E2 extracted in liquid-liquid extraction. Acetonitrile that was used in MIP preparation was not included as it would not make a stable liquid membrane. The results shown in Figure 5.5 indicate that more E2 would partition into hexane-ethyl acetate mixture (3:2) than in any other studied solvent. This was taken as the best organic acceptor solution. E2 has hydroxyl group that can contribute to hydrogen bonding in solvents such as ethyl acetate. This may explain why high E2 partitioning was obtained in hexane-ethyl acetate mixture. London dispersion forces increase with molar mass of a solvent. Linear molecules also results in high london forces compared to branched ones as this maximizes the interaction. These aspects

may explain why hexane was much better compared to cyclohexane. A mixture of hexane-ethyl acetate gave also better E2 binding onto MIP beads than toluene and pure hexane in the MMLLE-MIP unit.

5.2.3 Washing solvent

It should be noted here that the purpose of washing is not exactly the same as in cases where an aqueous sample is percolated through a MIP sorbent. In the latter case, during sample application, both the target analytes and matrix compounds are bound onto the MIP sorbent. Washing is then necessary to remove the matrix components bound non-specifically. In the present MMLLE-MIP, since binding of the analytes is very specific in the organic acceptor phase, there is no need of washing once the MIP particles are separated from organic phase. However, a washing solvent is necessary because after extraction, the whole acceptor phase is passed through the syringe filter where MIP particles are retained. In this process, other interfering compounds in the organic phase contaminate the filter so that washing becomes a necessary step before finally eluting the bound analytes from the MIP particles.

The washing solvent should remove weakly bound interfering substances without interfering with the binding of the target compounds. This means that the distribution coefficient of the interfering substances towards the MIP should be low whereas that of the analyte should be high (Pap *et al.*, 2003). Dichloromethane was chosen as the best washing solvent for triazines extraction as it gave much cleaner chromatograms and high enrichments after analyte desorption from the MIP particles. Other solvents

such as ethyl acetate, acetonitrile, butyl methyl ketone and toluene were tested too. Since the polymer was prepared in toluene, it was supposed to give the best washing. However, dichloromethane was found to be a better choice. Pap *et al.,* (2003) is also reported to have studied the influence of several organic solvents for selective washing on terbutylazine imprinted polymer sorbent and chose dichloromethane as the best solvent.

The washing solvent for E2 was optimised in the previous study (Nemulenzi *et al.,* 2008). Fresh organic acceptor hexane –ethyl acetate (3:2) was chosen as the washing solution.

5.2.4 Variation of the desorption solvent

Many desorption solvents of the analytes from the MIP have been reported. These include methanol (Pap *et al.,* 2003; Chapius *et al.,* 2003) acetonitrile (Turiel 2001), methanol-acetic acid (4:1) (Baggiani *et al.,* 2001). In the best case the desorption solvent elutes the bound analytes preferably in one single extraction and with minimal volume to avoid dilution of the analytes. Since the elution solvent might need to be evaporated to concentrate the sample before injection into the HPLC, an elution solvent with low boiling point would be more ideal.

Figure 5.6 shows the results on studying the type of desorption solvent from MIP particles. Water with its ability to make hydrogen bonds would be expected to disrupt the bonding between the polymer and triazine analytes and therefore give better extraction efficiencies. Since pure water alone was not efficient in desorbing the

triazines compared to acetonitrile and methanol, this does indicate that the MIP particles may not be wetted well enough by water (Claude *et al.*, 2008). 90% methanol in water was found to be the best solvent compared to methanol, water and acetonitrile. The weakness with 90% methanol in water is that it evaporates slowly therefore two or three days would be required to evaporate 3 ml to dryness.

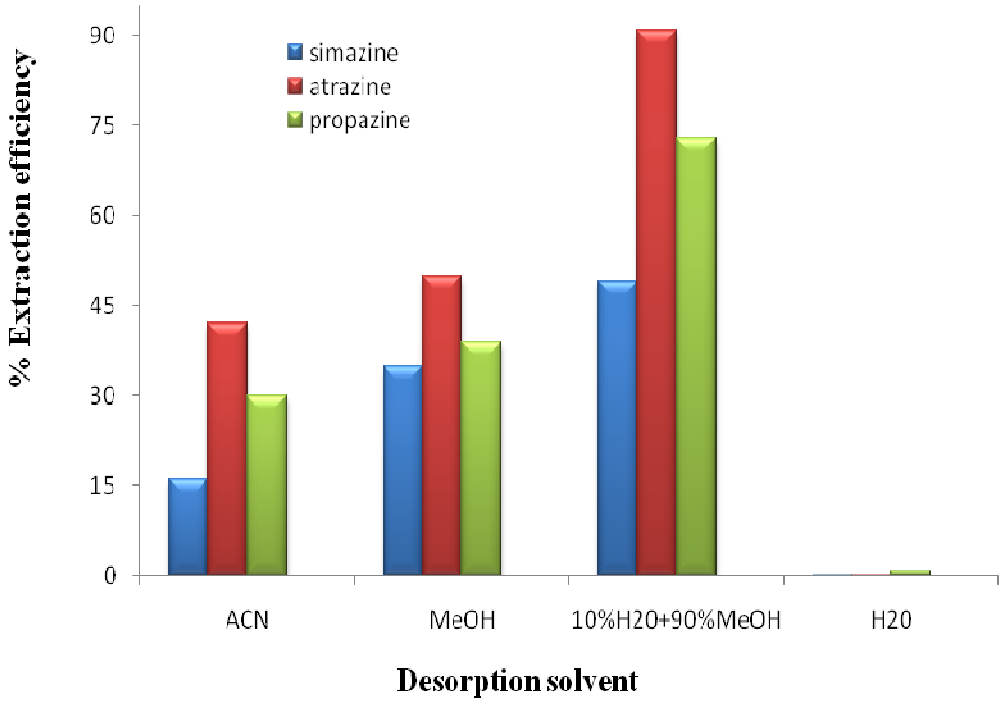


Figure 5.6: Influence of desorption solvent on extraction efficiency. Extraction time was for 60 minutes. 0.1 mg l^{-1} of triazine mixture spiked in deionised water was extracted with 10 mg of MIP in the organic acceptor solution (2.5 ml toluene). 2 ml of dichloromethane was used as a washing solution of MIP followed by desorption of the solvents.

It should be mentioned here that similar trend and results were observed during the E2 study. Therefore 90% methanol in water was chosen as the best eluting solvent. Besides giving higher extraction efficiencies, methanol solutions also gave cleaner chromatograms when compared to acetonitrile elution.

5.2.5 Varying the extraction time

The effect of varying the extraction time was studied for the triazine herbicides extraction only. The extraction time was taken as 60 minutes for the E2 extraction as it was optimized as explained in the experimental.

Figures 5.7 and 5.8 shows the results of varying the extraction time without MIP and with MIP particles respectively of the triazines extraction. The effect of varying extraction time was investigated from the donor side. The amount of triazines remaining in the donor side decreased and the subsequent amount of triazines extracted increased with extraction time. This is expected since the diffusion of triazines from the donor side of the extraction unit through the membrane into the bulk acceptor phase and subsequent binding to the MIP (where it is included) is time dependant. More time allows triazines to diffuse through the membrane into the acceptor leading to more binding onto MIP particles. It also allows the sample solution to be in contact with the membrane for longer periods (Cukrowska *et al.*, 2007). However, at around 90 minutes, there are signs of reaching a plateau especially for atrazine and propazine. 90 minutes was thus taken as optimum

extraction time since it was a compromise between high extraction efficiency and sample throughput.

It should be pointed here that 90 minutes is too long for the use of MMLLE – MIP technique for routine analysis. The slow mass transfer from the donor side onto the MIP particles can be attributed to the design of the extraction unit. Mass transfer occurs only from one side where the membrane is in contact with sample solution. A more miniaturized system is thus desirable that allows the fast movement of analytes from the donor onto the MIP particles. A hollow fibre membrane can thus be recommended with MIP particles in the lumen. This will give mass transfer from all sides. In liquid membrane extraction, hollow fibre modules are now seen as the module of choice because they give high enrichment factors (Jönsson *et al.*, 2008).

The inclusion of the MIP particles in the extraction protocol increased the amount of the triazines extracted from the donor. This was because the binding of the triazines onto the MIP particles maintained a high concentration gradient between the bulk acceptor and donor hence more triazines would diffuse from the donor to the acceptor through the hydrophobic membrane.

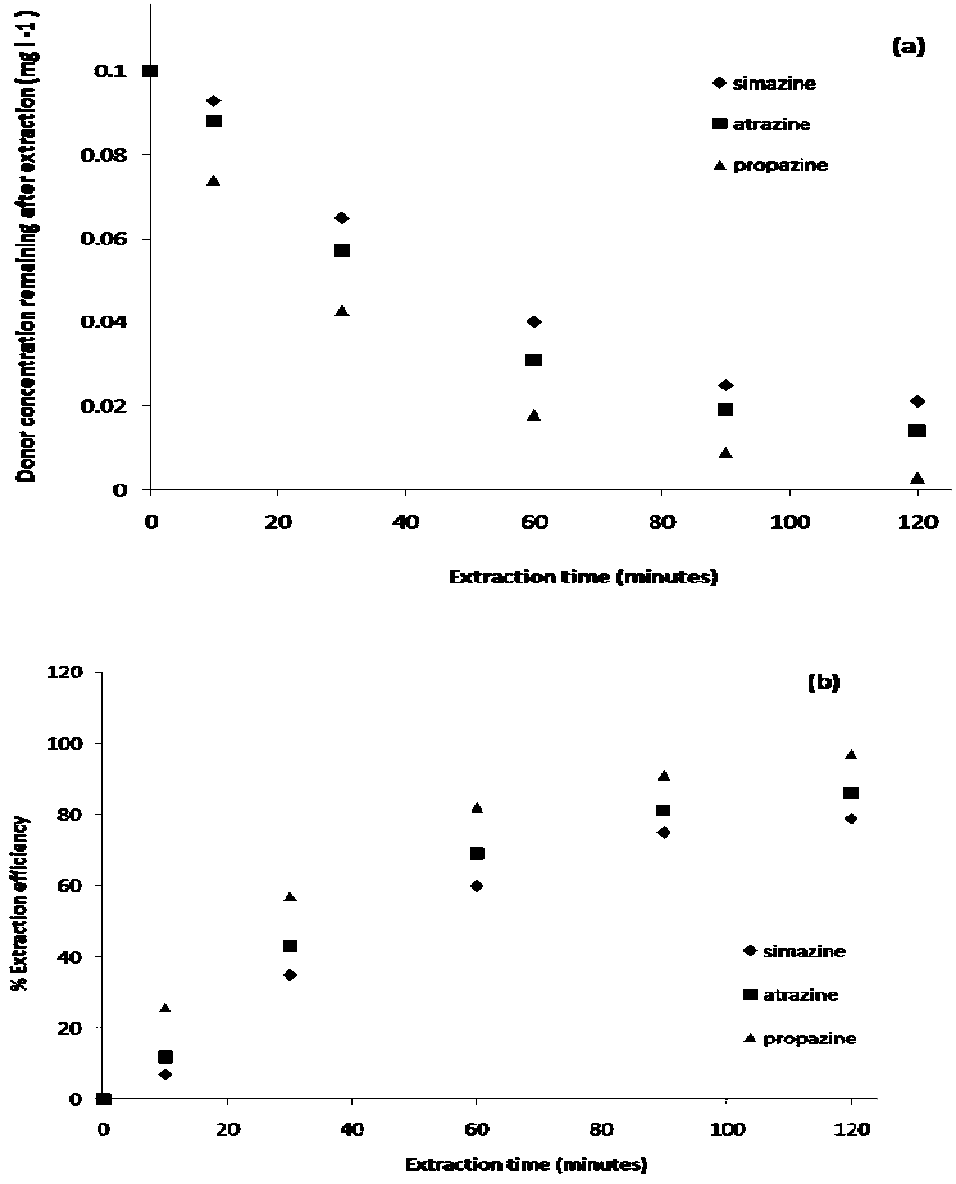


Figure 5.7: Influence of extraction time on the extraction efficiency without MIP particles in the acceptor phase. 0.1 mg l^{-1} of triazines mixture spiked in deionised water was extracted with 2.5 ml of toluene in the organic acceptor compartment. (a) The donor concentration remaining after extraction. (b) Percent extraction efficiency against extraction time determined from the donor side of the membrane.

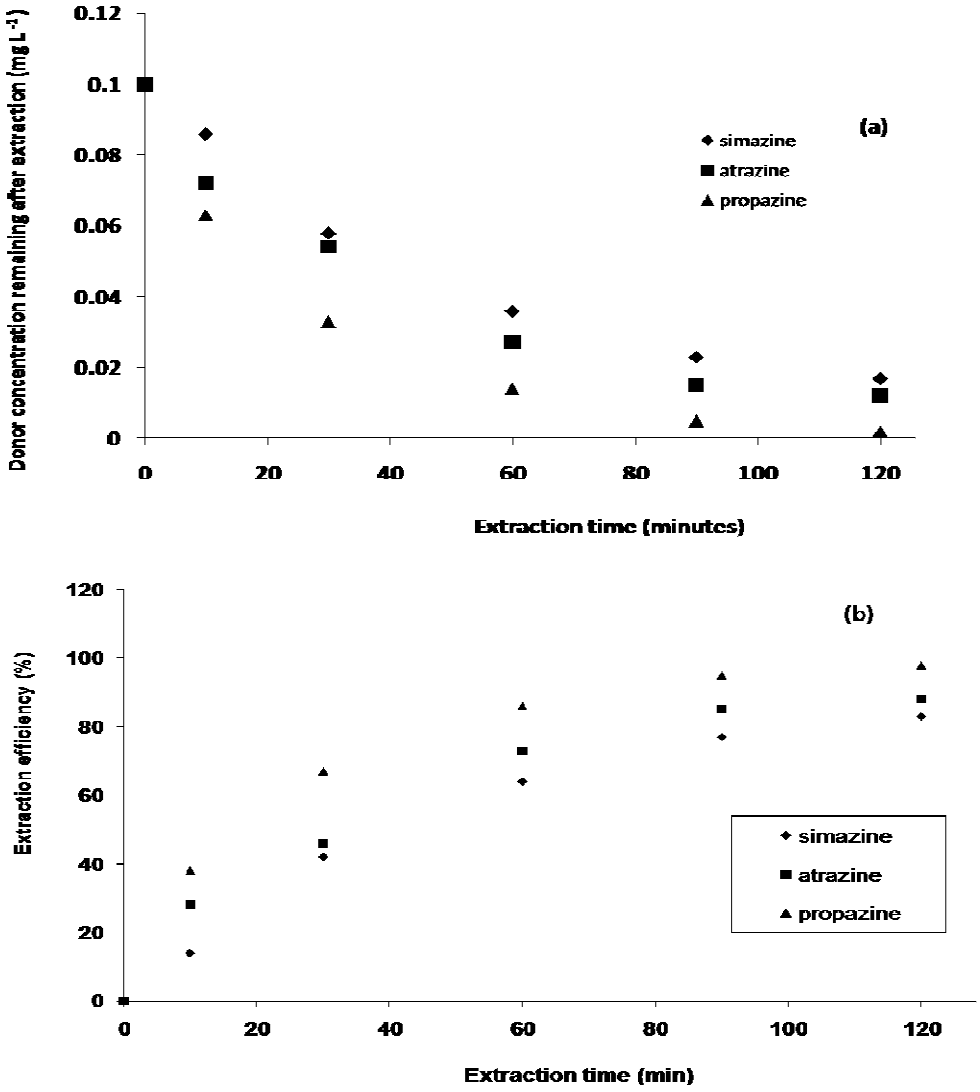


Figure 5.8: Influence of extraction time on extraction efficiency. 0.1 mg l^{-1} of triazine mixture spiked in deionised water was extracted with 10 mg of MIP in the organic acceptor solution (2.5 ml toluene). 2 ml of dichloromethane was used as a washing solution of MIP followed by desorption with 90% methanol in water. The extraction efficiency was monitored from the donor side of the membrane (see equation 4.3). (a) The donor concentration remaining after extraction, (b) percent extraction efficiency against extraction time determined from the donor side of the membrane.

After 90 minutes of extraction using MMLLE only, the extraction efficiencies were 75% for simazine, 81% for atrazine and 91% for propazine. The extraction efficiencies for MMLLE - MIP were 77% for simazine, 85% for atrazine and 95% for propazine (Table 5.1). These results prove that the presence of the MIP in the acceptor phase led to more triazines being extracted from the donor. The corresponding enrichments factors of the MMLLE – MIP extractions are shown in Appendix A1.

Table 5.1: Comparison of the MMLLE with and without MIP after 90 minutes of extraction

Compound	Concentration remaining in the donor (mg l^{-1})		% Extraction Efficiency	
	Without MIP	With MIP	Without MIP	With MIP
Simazine	0.025	0.023	75	77
Atrazine	0.019	0.015	81	85
Propazine	0.009	0.005	91	95

The extraction efficiency measured from the donor side (Figure 5.7b) at 90 minutes of extraction was compared to that measured in the acceptor after desorption from MIP particles in the MMLLE – MIP extraction and in the bulk acceptor in case of MMLLE only.

The comparison is shown in Table 5.2. From the table it can be shown that a small percent of triazines compounds remain in the bulk acceptor solution in the case of MMLLE – MIP combination hence lower extraction efficiencies (E_A) after desorption when compared to MMLLE only. The differences between the extraction efficiencies with and without MIP shown in Table 5.2 at 90 minutes of extraction, suggest that at this time, binding of the triazine to MIP was the rate limiting step. This is supported by Figures 5.7b and 5.8b. At the extraction time between 0 and 60 minutes, the extraction is somewhat linear. This means that in this region, diffusion of triazines from the acceptor solution was controlling the mass transfer in the MMLLE – MIP technique.

However the combination still remains superior to MMLLE extraction only because cleaner chromatograms are obtained from the MIP desorption (Figures 5.15 and 5.16). The triazines remaining in the bulk acceptor does not interfere with next extraction since fresh acceptor solution is used each time. Increasing the amount of the MIP in the acceptor phase could however reduce the remaining amount hence improve extraction efficiency.

Table 5.2: Comparison of the extraction efficiency measured from the donor side of the membrane (E_D) and from the acceptor side (E_A) at optimised conditions (see Figure 5.15). Sample concentration: 0.1 mg l^{-1} of triazine in deionised water. E_A^* was after desorption of the triazines from the 10 mg of MIP particles whilst E_A was obtained from the bulk acceptor solution because the MIP was not included.

Compound	**% Extraction efficiency of MMLLE with MIP**		**% Extraction efficiency of MMLLE without MIP**	
	E_D	E_A^*	E_D	E_A
Simazine	77	53	75	63
Atrazine	85	74	81	76
Propazine	95	65	91	83

5.2.6 Permeation studies

The permeation studies depict the movement of the analytes from the aqueous sample into the acceptor phase. Figure 5.9 (a) and (b) shows the typical permeation study of the three triazines obtained with and without the MIP respectively. The results do indicate that diffusion of the analytes into the acceptor phase follow a first order reaction kinetics. The transport rate is proportional to the initial concentration (not shown).

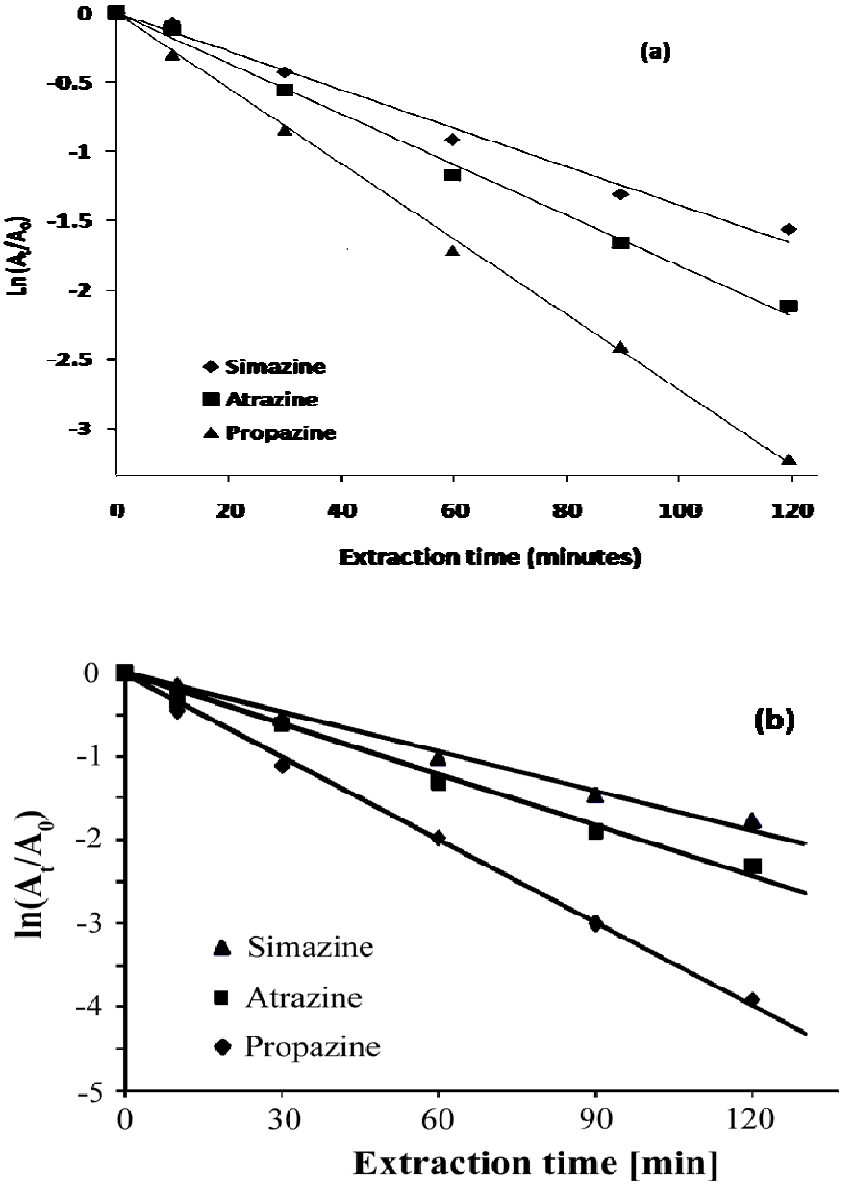


Figure 5.9: Relative triazine concentration in the donor compartment as a function of extraction time. 0.1 mg l^{-1} of triazine mixture spiked in deionised water was extracted, (a) with 2.5 ml of the acceptor solution and (b) with 10 mg of MIP plus organic acceptor solution (2.5 ml toluene). 2 ml of dichloromethane was used as a washing solution of MIP followed by desorption with 90% methanol in water.

From the permeation studies, the slopes (the permeation constant *k*) were calculated, $t_{1/2}$ was obtained by using equation 4 and Table 5.3 was obtained as a summary. Propazine displayed the fastest transport followed by atrazine, and simazine showed the lowest rate. This is expected since propazine is the most hydrophobic triazine (Appendix A2) and will partition into toluene more easily compared to atrazine and simazine (Table 1.2), and simazine is the least hydrophobic species. Chimuka *et al.*, (1997) reported similar permeation results on the study of the transport of nitrophenols across a supported liquid membrane technique. The most hydrophobic nitrophenol was extracted much higher compared to others. In another recent study on fungicides (Michael *et al.*, 2009), the most hydrophobic fungicides gave the highest mass transfer with dihexyl ether as the membrane solvent.

Table 5.3: Comparison of the permeation studies measured from the donor side of the membrane with and without MIP. The experiments were performed at optimized conditions (see Figure 5.15) and the samples consisted of 0.1 mg l^{-1} of total triazine in deionised water.

Compound	Permeation constant, k (min^{-1})		Half life (min)	
	MMLLE	MMLLE-MIP	MMLLE	MMLLE-MIP
Simazine	0.013	0.016	50	44
Atrazine	0.018	0.020	39	34
Propazine	0.028	0.033	25	21

Comparison of permeation studies with and without MIP in the acceptor phase (Table 5.3 above) show that the presence of MIP enhanced the transport for all compounds to a certain extent, by removing the compounds from the acceptor phase and thus maintaining a concentration gradient. The difference in half lives between that with MMLLE – MIP and MMLLE are not significant. The half life differences are between 4 to 6. This again suggests that an additional amount of MIP could shorten the half life for triazines in MMLLE – MIP combination.

5.2.7 The effect of donor concentration

During real sample extraction, different concentrations of triazines will be encountered. Therefore it is important to study how the donor concentration of triazines may affect the extraction efficiencies and enrichments factors. The effect of the donor concentration was investigated by varying the donor concentrations (2.5, 5, 10 and 30 $\mu g\ l^{-1}$) and extraction efficiency calculated.

The driving force in liquid membrane is the difference between the donor analyte concentration and acceptor analyte concentration (Chimuka *et al.,* 2004). A bigger concentration gradient will therefore provide greater enrichment and extraction efficiency. The results in Figure 5.10 showed that the extraction efficiency somewhat is dependant on the donor concentration. However, this was not very much pronounced. At lower concentrations where real environmental values are expected, the extraction efficiency was almost constant. The same trend is observed on the enrichments factors shown in Appendix A3.

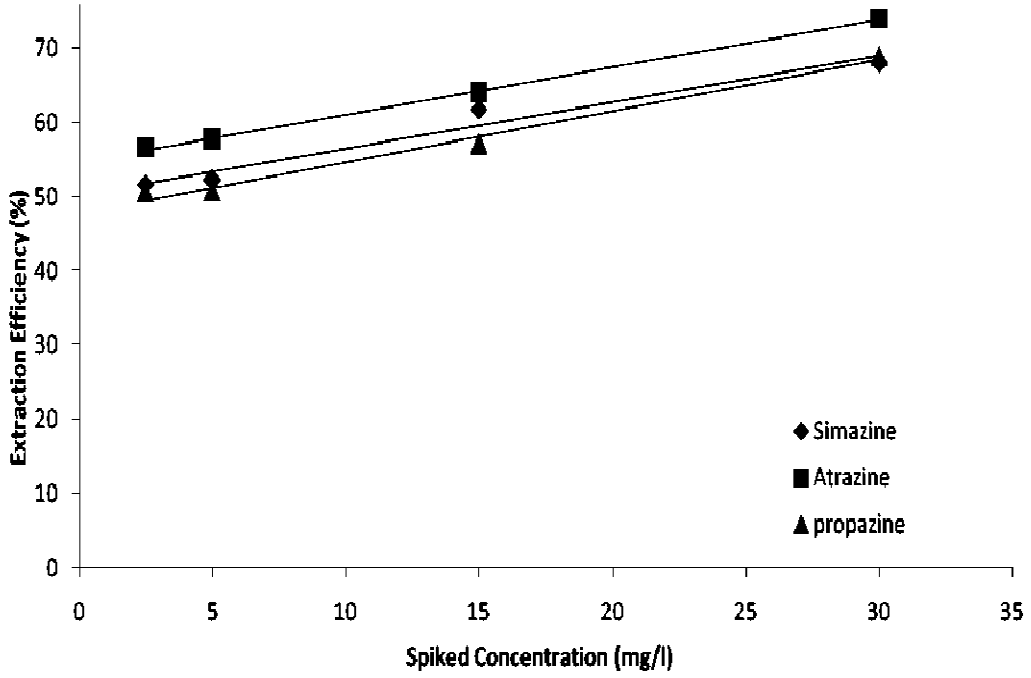


Figure 5.10: The influence of donor concentration on extraction efficiency. *n* = 3, average coefficient of variance (CV) across the four concentration studied are; simazine = 8%, atrazine = 5% and for propazine = 7%, see Table 5.4.

The slight donor concentration dependence could be due to slow binding of MIP particles in the acceptor phase. This could be reduced by increasing the amount of MIP particles or changing the configuration so that it improves the mass transfer as discussed. Another possibility is to allow the system stand for additional minutes after extraction so that all analytes still in the membrane can diffuse into the bulk acceptor solution and bind to MIP. In liquid membrane extraction alone, slow mass transfer between the membrane and bulk acceptor solution referred to as membrane memory effect gives rise to extraction efficiency to be donor concentration dependent (Milotis *et al.,* 1996). This membrane memory effect is generally more pronounced at

lower concentrations and for most hydrophobic compounds. In Figure 5.10, it seems the extraction efficiency dependence on the donor concentration is more pronounced at high concentration. This suggests the problem is not only slow mass transfer between the membrane and the bulk acceptor solution. Slow binding of triazines to MIP particles could be part of the problem. Increasing the amount of MIP to increase the binding sites could be the best option to make the MMLLE – MIP technique less donor concentration dependent.

Table 5.4: The distribution of the extraction efficiencies between the washing solution (dichloromethane, E_W), organic acceptor (toluene, E_O) and elution solvent (methanol water, E_A). 30 mg l^{-1} of triazine mixture spiked in deionised water was extracted, (a) with 2.5 ml of the acceptor solution and (b) with 10 mg of MIP plus organic acceptor solution (2.5 ml toluene). 2 ml of dichloromethane was used as a washing solution of MIP followed by desorption with 90% methanol in water.

Compound	Lettuce vegetable extract			Apple fruit extract		
	E_A	E_w	E_o	E_A	E_w	E_o
Simazine	68	3	8	55	3	7
Atrazine	77	5	6	63	7	6
Propazine	69	4	4	60	3	5

Table 5.4 above shows the distribution of the extraction efficiency between the washing solution (dichloromethane), organic acceptor (toluene) and elution solvent

(methanol /water). When the individual extraction efficiencies are added, the total value is less than 100% and this support the theory of memory effects.

The percentage R.S.D values for the experiment on varying the donor concentration shown in Table 5.5 below. The % RSD values varied between 5 and 10% for simazine, 1 and 7% for atrazine, 3 and 14% for propazine. The % RSD values are independent of sample concentration. In most extractions, the % RSD values tend to be high at lower concentrations due to losses or memory effect especially in liquid membrane extraction. It should be noted here that these % RSD values are a combination of two extraction process. Therefore although the recommended % RSD values are those that fall below 5%, the range of 1 to 14% in this case would still be acceptable.

Table 5.5: The % RSD values of the extraction efficiencies (E_D) obtained when various concentrations were extracted.

Concentration ($mg\ l^{-1}$)	% RSD		
	Simazine	Atrazine	Propazine
2.5	7	7	3
5	10	6	5
15	10	1	14
30	5	5	5
Average	8	5	7

5.2.8 The effect of humic substances on the extraction efficiency

The effect of humic acids was studied on extraction efficiency using triazines herbicides as the model compounds. Different concentrations (0, 5, 10 and 20 mg l^{-1}) of humic substances were added to deionised water spiked to a total final concentration of 30 µg l^{-1} triazines. This was done to understand the possible interactions between humic substances and target compounds. Interactions may affect the transport of polar organic compounds such as triazines in system.

Table 5.6 below shows the results of the effect of humic substances on the extraction efficiencies. The results indicate that the humic substances affected the transport of triazines as extraction efficiency slightly decreased in the presence of humic substances (HSs). However increasing the humic substances concentration from 5 to 20 mg l^{-1} did not affect the extraction efficiency. The low extraction efficiency in humic substances when compared to pure water could be attributed to interactions between the humic substances and triazines in the donor phase.

In solid phase extraction HSs have been noted to give a huge peak at the beginning of the chromatogram as they are also extracted and co-eluted by the most common non-selective sorbents (Shen *et al.*, 1994, Megersa *et al.*, 1999). For the purpose of comparison, a chromatogram obtained when triazines were extracted from soil extract using SPE is shown in Figure 5.11. In this study there was no such huge peak (Figure 5.12) signifying that MIPs selectively extracted triazines only. Humic substances did not bind to the MIP since clean chromatograms were obtained. Liquid membrane

alone especially supported liquid membrane extraction can prevent the extraction of HSs across to the acceptor phase. This was demonstrated by Mergesa *et al.*, (1999). In this study, SLM extraction technique was compared to SPE with C_{18} sorbent. A huge peak at the beginning of the chromatogram from SPE injection was obtained while SLM extraction technique gave clean chromatograms. It is therefore expected for the MMLLE – MIP combination to give cleaner chromatograms, since the HSs will be prevented from reaching the MIP particles in the acceptor solution.

Table 5.6: The effect of humic acid on the extraction efficiency of triazine herbicides. (% RSD values are included in brackets, $n = 3$).

	% Extraction efficiency (Bound to MIP) Varying the Concentration of Humic Acid (mg l^{-1})			
	0	5	10	20
Simazine	68 *(5)*	56 *(4)*	55 *(10)*	48 *(15)*
Atrazine	74 *(5)*	60 *(6)*	59 *(3)*	60 *(8)*
Propazine	69 (5)	51 *(4)*	50*(12)*	54 *(9)*

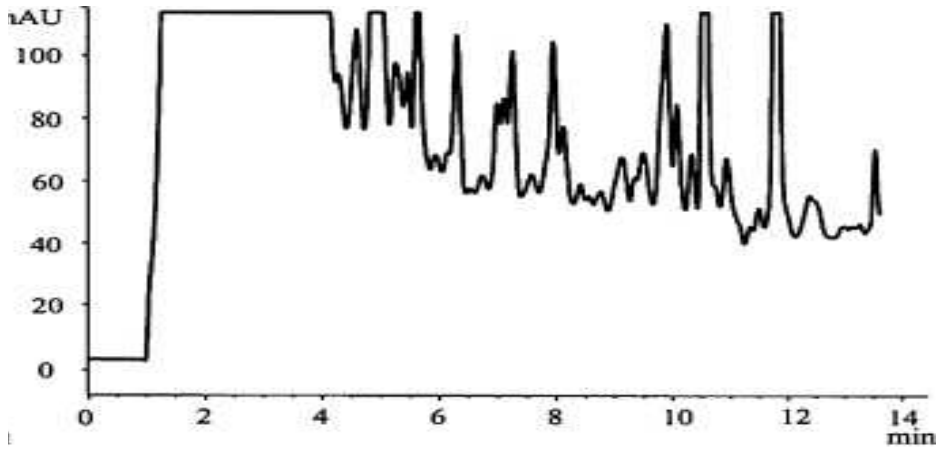


Figure 5.11: Chromatogram obtained after the injection of a soil extract containing 20 ng g^{-1} of triazines. Compounds extracted were atrazine, simazine, terbutylazine. Note the huge peak at the beginning of the chromatogram (Chapius *et al.*, 2004).

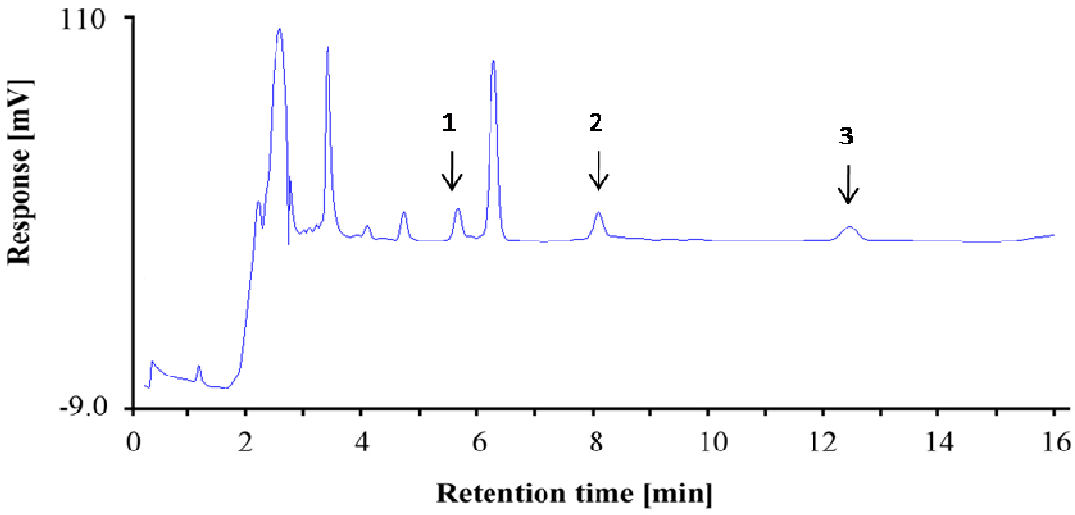


Figure 5.12: Chromatogram obtained following MMLLE – MIP extraction of a sample spiked with 20 mg l^{-1} humic substances. The sample was spiked with humic substances and 30 µg l^{-1} triazines. Note the absence of a huge peak at the beginning of the chromatogram as in most SPE. 1 = simazine, 2 = atrazine and 3 = propazine.

5.2.9 Influence of ultrasound assisted extraction on rebinding of E2 on the MIP particles

Avila *et al.,* (2007) is reported to have used ultrasound assisted extraction to accelerate silylation of triterpenic compounds by enhancement of the reaction kinetics. Thus, the 2 hours required as reaction time with the conventional procedure can be shortened by ultrasound influence. The result of the study showed that only 5 minutes were required to complete silylation of the triterpenic compounds. It was thus anticipated that perhaps the slow binding kinetics of E2 onto MIP beads could be accelerated by ultrasound energy.

The results of such study are shown in Figure 5.13. The enrichment factor in this case increased with ultrasound time until after 20 minutes where there was a decline. These results may suggest that where slow kinetics occurs in the MIP, ultrasound energy could be used. The sharp decline after twenty minutes may due to temperature increase in the solution as it was not controlled. E2 binding onto MIP particles involves weak bond formation so it is an endothermic reaction. Increasing temperature will therefore not favour E2 binding. An ultrasound time of 20 minutes was thus taken as optimum.

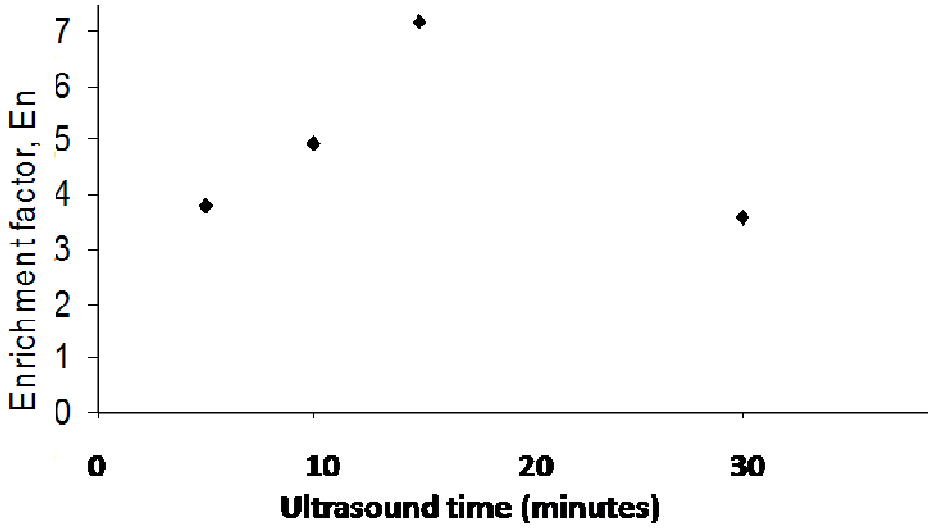


Figure 5.13: The effect of ultrasound on extraction process. A 200 watt equipped with a 4 mm diameter probe was used with probe depth of about 1cm. The whole acceptor phase with 30 mg of MIP was used.

5.2.10 The validity of the developed technique and comparison to LME

The validity of the developed LM – MIPSE was investigated by using triazines as model compounds. A 5 µg l^{-1} reference material containing ametryn, prometon, prometryn, propazine, simetryn, simazine and terbutryn was spiked into waste water and deionised water and spiked samples were extracted. Figure 5.14 presents the extraction efficiencies obtained.

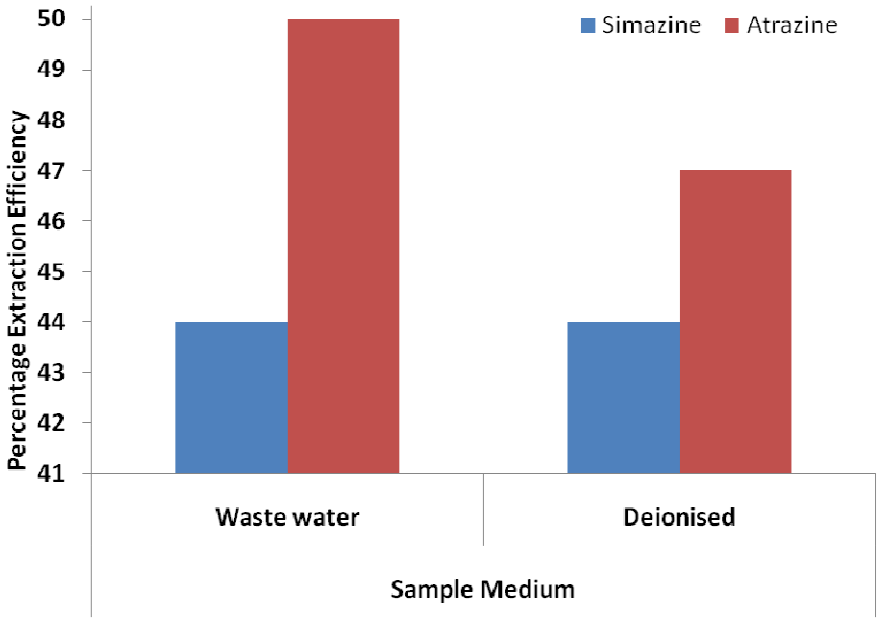


Figure 5.14: The percentage extraction efficiencies obtained when wastewater and deionised water was spiked with 5 µg l^{-1} reference standard was extracted at optimised conditions. Only simazine and atrazine were quantified.

The results were compared to those obtained when 5 µg l^{-1} non reference standards was extracted from deionised water. The extraction efficiencies obtained when deionised water spiked with 5 µg l^{-1} non-reference standard was extracted are 52%, 57% and 50% for simazine, atrazine and propazine respectively. These extraction efficiencies were used to calculate the concentration of the extracted reference material by rearranging equation 4.3 (Table 5.6). The results indicate that developed extraction technique can be reliably used to determine real samples since spiked concentrations were close to the determined ones. The accuracy ranged from 80 to 102% for simazine and atrazine while propazine was not quantified since it was not in the reference material. The use of reference materials is generally one of the accepted

ways to validate the method. Other methods include comparison with accepted extraction method and inter - laboratory comparisons.

Table 5.7: The table shows the calculated concentrations of extracted reference materials using extraction efficiencies obtained when a normal 5 µg l^{-1} standard was extracted. % RSD values are included in brackets.

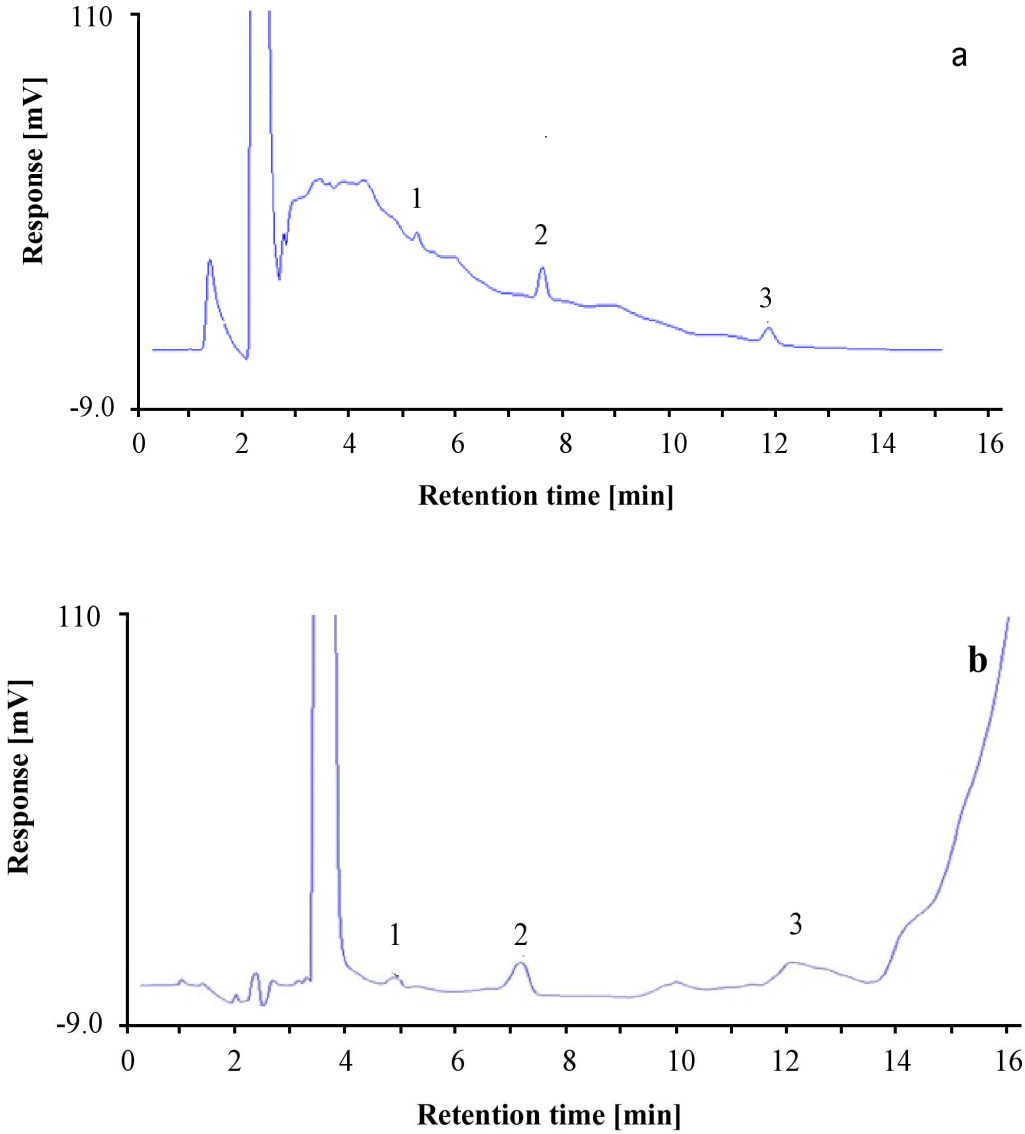
	Simazine			**Atrazine**		
	Spiked (5µg l^{-1})	Determined (5µg l^{-1})	Accuracy (%)	Spiked (5 µg l^{-1})	Determined (5 µg l^{-1})	Accuracy (%)
Ref material spiked in deionised water	5	5.1 *(4)*	102	5	4.1 *(2)*	82
Ref material spiked in waste water	5	4.4 *(4)*	88	5	4.0 *(7)*	80

5.3 APPLICATION OF THE DEVELOPED MMLLE – MIP TECHNIQUE TO SPIKED REAL SAMPLES

5.3.1 Extraction of food samples spiked with triazines herbicides

The selectivity of the MMLLE-MIP technique was demonstrated by extracting spiked vegetable and fruit extracts. The obtained chromatograms are given in Figures 5.15 and 5.16. In Figure 5.16a, a direct injection of the vegetable extract spiked with 100 µg l^{-1} gave very small triazine peaks and a large peak due to matrix effects. Injection

of the organic acceptor solution after MMLLE extraction of 30 µg l^{-1} of the triazine mixture spiked in a vegetable extract only did not improve the chromatogram (Figure 5.15b). A combination of MMLLE-MIP (Figure 5.15c) gave a clean chromatogram and well distinguishable peaks.



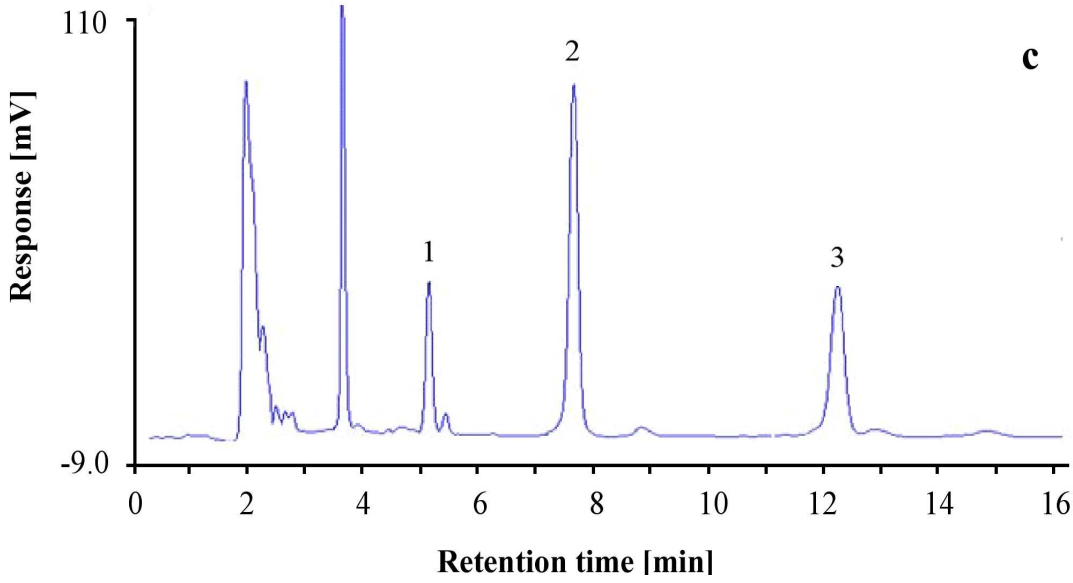
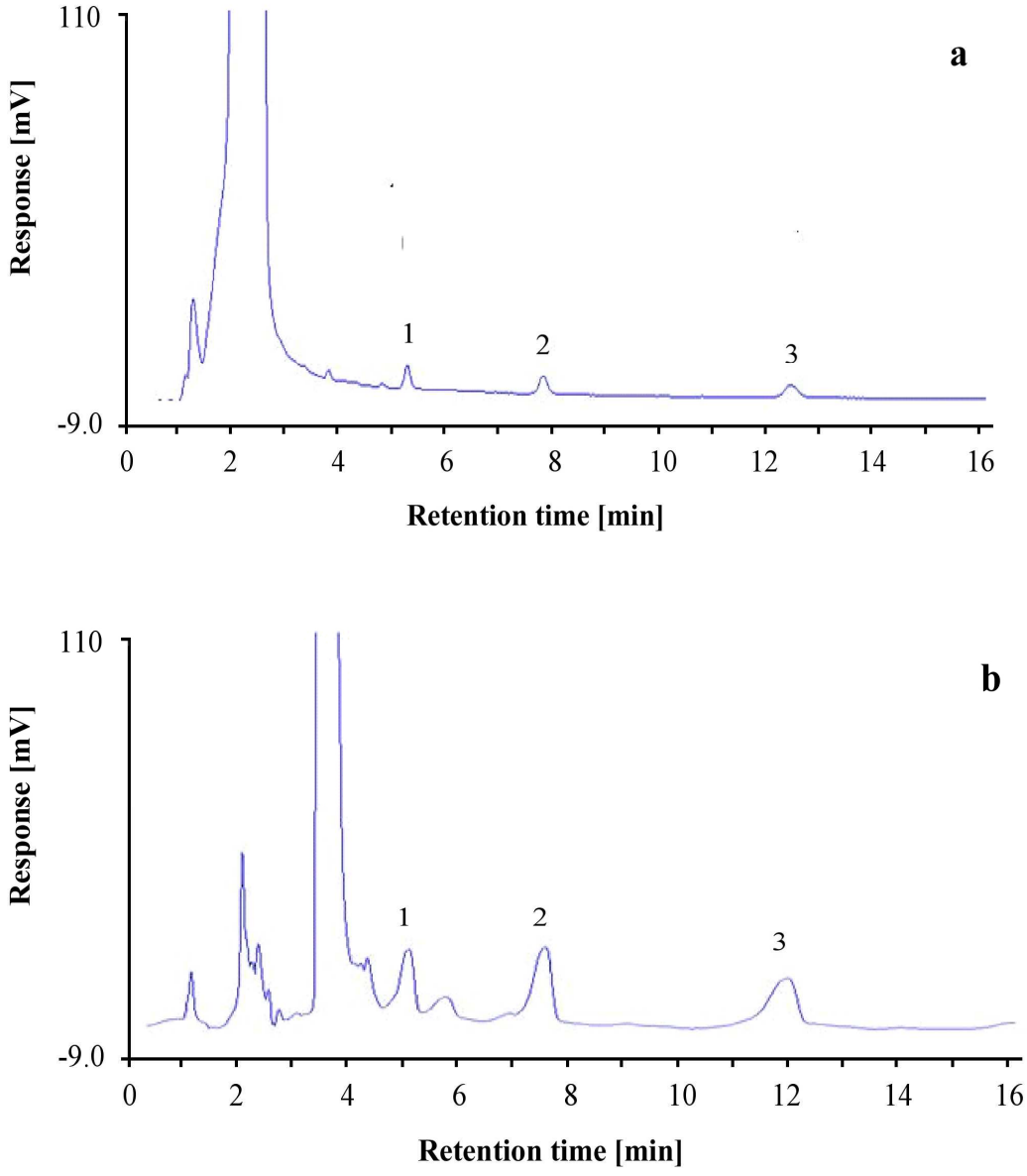


Figure 5.15: Chromatograms obtained after direct injection of lettuce extract spiked with 100 µg l^{-1} of the triazine mixture (a), MMLLE extraction of lettuce extract spiked with 30 µg l^{-1} of the triazine mixture (b), MMLLE-MIP extraction of same vegetable extract spiked with 30 µg l^{-1} of the triazine mixture (c). Experiments performed under optimised conditions of 10 mg of MIP in toluene as part of the acceptor solution, 2 ml of dichloromethane as a washing solution of MIP followed by desorption with 90% methanol in water. Extraction time was for 90 minutes. 1 = simazine, 2 = atrazine, 3 = propazine

A similar observation was also observed in the extraction of apple extract (Figure 5.14). In the extraction of apple extract, MMLLE–NIP was also compared. This also did not result in clean chromatograms. The clean chromatograms obtained in the combination of MMLLE – MIP for both lettuce and apple extract demonstrate the selectivity potential of the developed technique. Plant extracts generally have high

matrix because a lot of macromolecules such as proteins, carbohydrates and small organic compounds are in high concentration. Most common extraction techniques (e.g SPE, LLE etc) require a clean up step before final determination. Alternately a selective determination such as mass spectroscopy (MS) is employed to be certain of the identification.



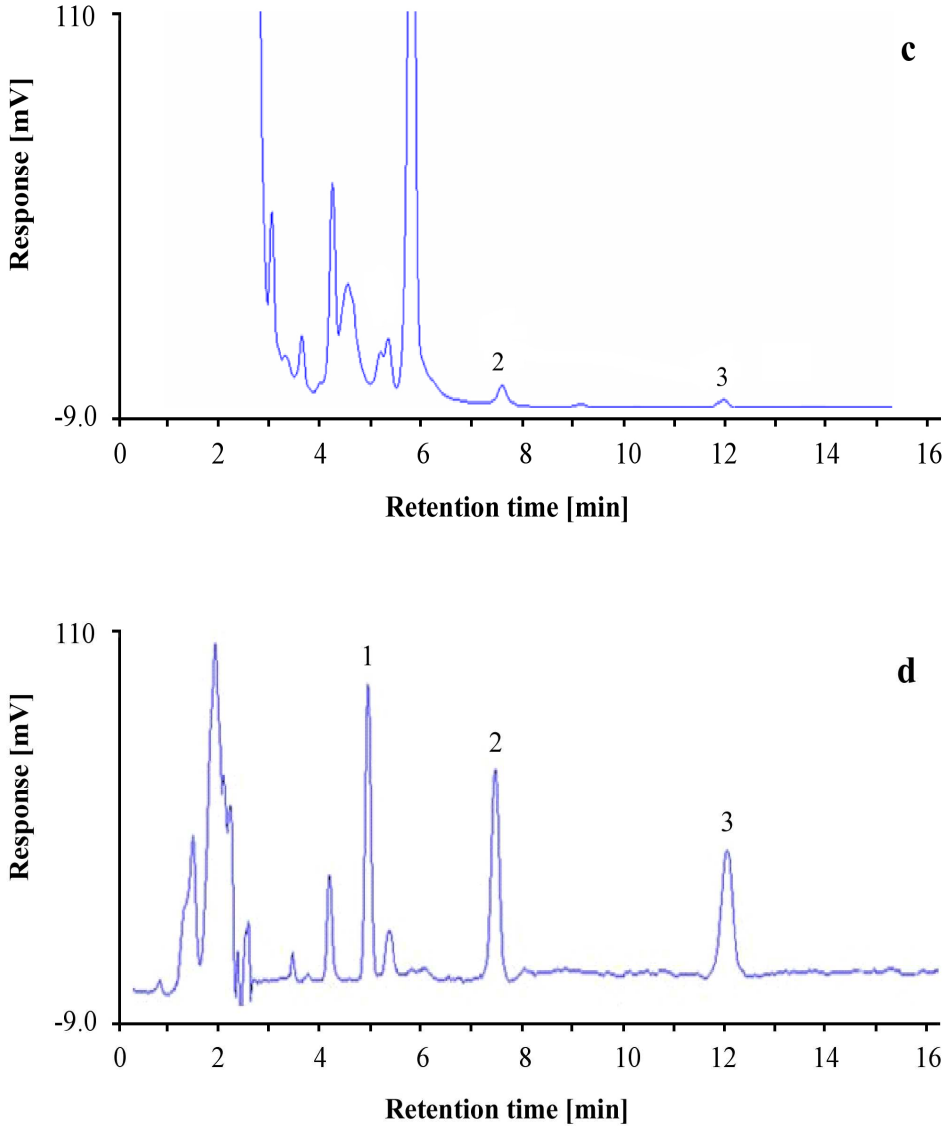


Figure 5.16: Chromatograms obtained after direct injection of apple extract spiked with 100 µg l^{-1} of the triazine mixtures (a), MMLLE extraction of apple extract spiked with 30 µg l^{-1} of the triazine mixture (b), MMLLE-NIP extraction of same apple extract spiked with 30 µg l^{-1} of the triazine mixture (c) MMLLE-MIP extraction of same apple extract spiked with 30 µg l^{-1} of the triazine mixture (d). For experimental conditions, see Figure 5.14.

Other researches have reported combination of different techniques in order to improve the selectivity (Khrolenko *et al.,* 2002; Thordasson *et al.,* 2000; Bjarnasson *et al.,* 1999). Khrolenko *et al.,* 2002 combined SLM –SPE in extraction of triazines from orange juice. The combination resulted in improved chromatograms that lowered detection limit. Thordason *et al.,* (2000) combined SLM – Immunoassay for determination of 4 – nitrophenol from spiked water solutions as well as spiked waste water sample. The combination proved useful when dealing with difficult matrices. Ferrer *et al.,* (1999) used a strong- anion exchange prior to C_{18} during trace enrichment of pesticides to remove humic substances. Once again Ferrer *et al.,* (1999) combined SPE and MIP for the extraction of triazines from tap water. The SPE (C_{18}) on its own could not give a good extraction but the inclusion of the MIP greatly reduced the humic peak but low recoveries (10 – 40%) were obtained.

Bjarnasson *et al.,* (1999) combined C_{18}-SPE with MIPSE. In this online combination samples consisting of humic substances and plant extracts were extracted and trapped on the C_{18} column. The trapped compounds were then eluted with an organic solvent to the MIPSE column. In MIPSE, the matrix compounds passed through unretained to the waste while the retained analytes were further eluted by injecting a plug of water and analysed on HPLC with UV detection. The combination showed remarkable selectivity. HSs were not retained by the MIPSE and much improved chromatograms were obtained from plant extracts. Cacho *et al.,* (2003) is also reported to have combined NIP – MISPE in extraction of triazines from vegetable extracts. A one step MISPE did not give clean chromatograms, hence a combination of NIP – SPE which

improved the selectivity of the extraction. Therefore for difficult samples such as plant extracts and sediments with high matrix samples, the trend is to combine two techniques. The developed MMLLE – MISPE therefore offers an alternative of these combinations.

Table 5.8 shows a comparison of extraction efficiency and enrichment factors for the Triazine compounds obtained in vegetable and apple extracts.

Table 5.8: Comparison of extraction efficiency (E_A) and enrichment factor (E_n) after extraction of 0.03 mg l^{-1} of triazine mixture spiked in lettuce and apple extracts. The experiments were performed at optimized conditions. Values in brackets are percentage relative standard deviations.

Compound	Lettuce vegetable extract		Apple fruit extract	
	E_A	E_n	E_A	E_n
Simazine	53 (7)	40	55 (5)	42
Atrazine	60 (10)	43	63 (15)	45
Propazine	55 (8)	42	60 (4)	43

The results show that the extraction process is independent of sample matrix. The obtained extraction efficiencies are generally low compared to those recommended in SPE techniques. Extraction efficiencies above 80% are recommended but in our case it ranged from 53 to 60%. This is understandable since difficult samples were

involved. Further in LME technique, it is normal to have extraction efficiencies lower than 80%. This is because extraction is governed by flow rate or stirring rate among other parameters. What is important therefore is the enrichment factor. This should allow detection of desirable concentrations in the sample. Further extraction efficiency should be constant at specified extraction conditions. The detection limit of MMLLE – MIP technique for plant extracts is shown in Table 5.9. This shows that it is possible to detect triazines at trace levels even in complex samples.

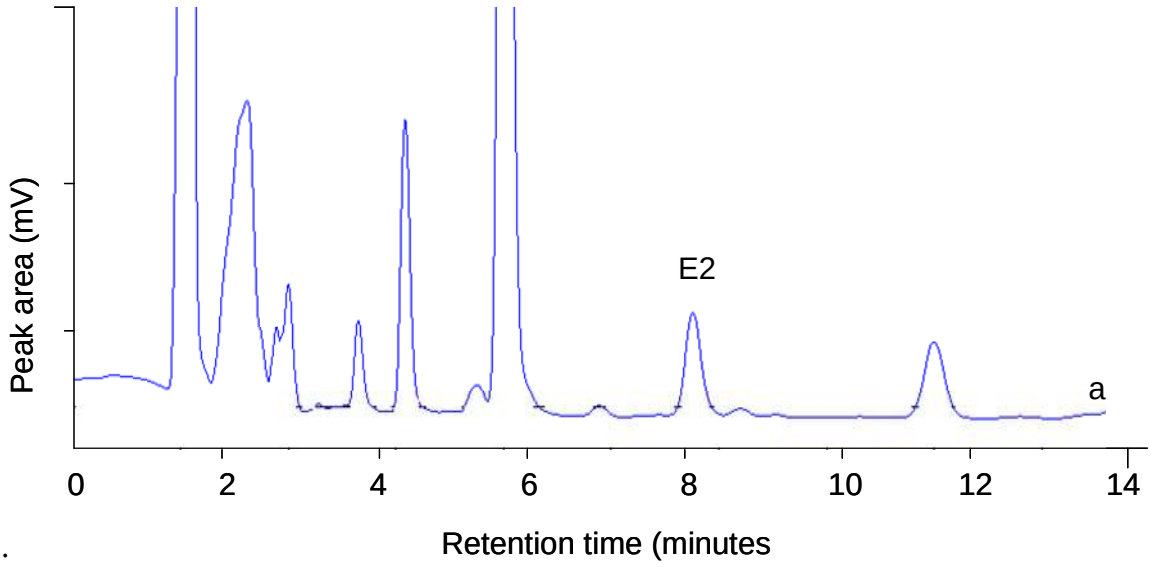
Table 5.9 The detection limit of the MMLLE – MIP technique for lettuce and fruit extracts calculated form the detection limit of direct injection of the standard and enrichments factors shown in Table 5.7 above.

Compound	Detection Limit (DL)		
	Direct injection ($\mu g\ l^{-1}$)	In lettuce vegetable extract ($\mu g\ l^{-1}$)	In apple fruit extract ($\mu g\ l^{-1}$)
Simazine	10	0.25	0.25
Atrazine	10	0.23	0.22
Propazine	15	0.36	0.35

5.3.2 Extraction of waste water samples spiked with E2.

The selectivity of the LM-MIP technique for E2 was demonstrated by extraction of spiked wastewater. Clean chromatograms were also obtained besides the fact

that wastewater has a lot of organic matrices that have high partition coefficients in the acceptor phase. A comparison of selective with the organic acceptor solution only and with incorporation of MIP particles was also compared for wastewater (Figure 5.17). Clean chromatograms highlights the benefits of incorporating MIP beads in the organic acceptor solution. In this case clean-up occurs simultaneously in the acceptor phase with extraction of the analyte from the donor phase. The organic acceptor alone did not yield clean chromatograms. The results are similar to those obtained for the extraction of triazines from food samples. Because of poor detection limit of E2 on the HPLC-UV system used, lower concentration could not be extracted. Only 100 and 500 µg l^{-1} was extracted. The enrichment factors were around 9. For wastewater influent, the enrichment factors were around 6 much lower than the effluent (9) perhaps due E2 binding to organic matter in the sample.



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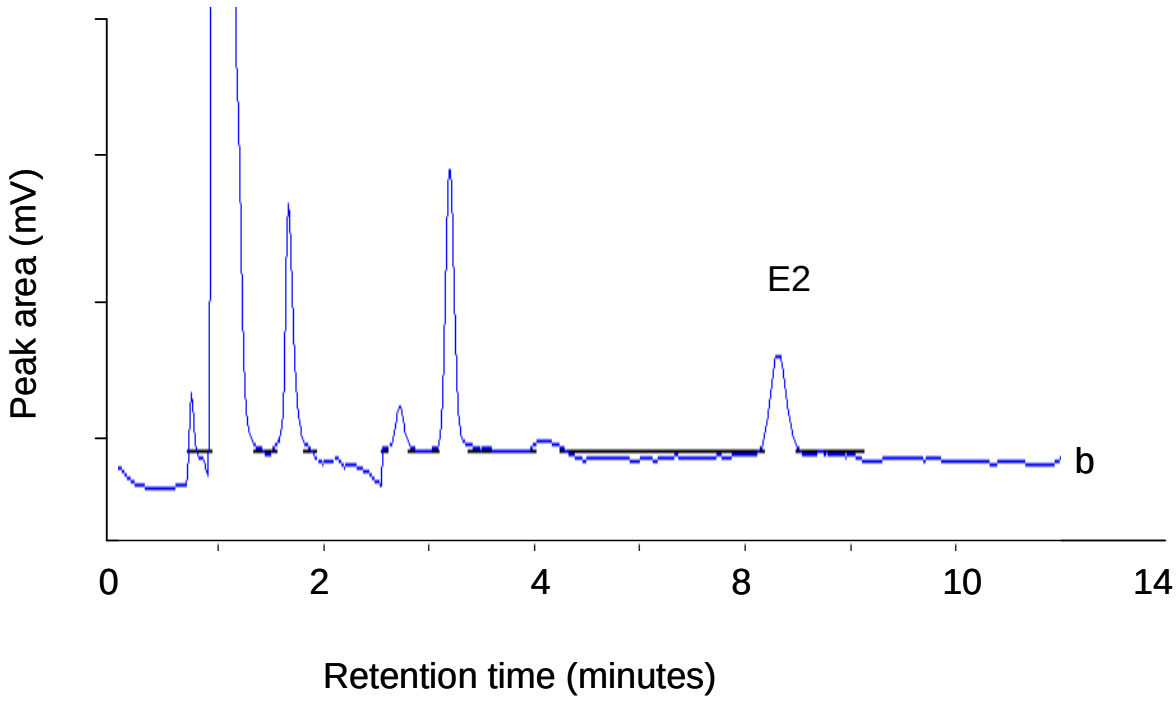


Figure 5.17: Chromatogram obtained after MMLLE extraction of wastewater effluent spiked with 500 µg l^{-1} E2, (a) without MIP particles and (b) with MIP particles in the acceptor. Extraction time was for 60 minutes and 3 ml acetonitrile was used to desorb E2 from the MIP beads.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Solid-phase extraction on conventional sorbents such as C_{18}, has been widely applied for the isolation and trace enrichment of organic contaminants from complex samples. SPE has been widely criticised for lack of selectivity. The increased availability of selective sorbents has easily overcome some of the drawbacks of C_{18}. However the use of a single extraction technique has also proved inadequate to extract and preconcentrate target analytes from complex samples. The trend is to combine two techniques in order to produce synergism with high selectivity as shown in this dissertation.

The potential of the macroporous membrane liquid- liquid extraction (MMLLE) and molecularly imprinted polymers solid phase extraction (MISPE) combination in a single extraction technique has been developed and demonstrated for extraction of plant extracts and wastewater. Triazine hebicides (simazine atrazine and propazine) were extracted and preconcentrated from plant extract samples while 17 β – estradiol (E2) was extracted and pre-concentrated from wastewater. The combination of MMLLE –MIP has great potential in extraction of complex samples because it has shown high selectivity even in the presence of humic substances and complex matrices. The use of standard reference material validated the accuracy of the new technique. Good enrichment factors and low detection limit, in regions of single $\mu g\ l^{-1}$ in complex plant extracts were obtained for triazines herbicides although for E2 low enrichments factors were obtained. The low enrichments factors for E2 was due to poor binding kinetics and high HPLC-UV detection limits..

A major advantage of the MMLLE – MIP technique is that extraction and clean up occurs in a single step. In common extraction techniques these are two separate steps, thus consuming a lot of time and organic solvents. The developed technique for extraction of organic pollutants also uses less organic solvents compared to conventional SPE and hence makes it more environmental friendly.

6.2 RECOMMENDATIONS

The developed technique need to be applied to a variety of samples with differing sample matrices in order to test for its versatility. The new technique need to be applied to real samples and compared to known techniques. This is important as part of the marketing strategy. It has to be shown beyond doubt that it can complement existing techniques. The optimised extraction time for triazines herbicides is 90 minutes. This is too long for routine analysis where many samples are to be processed. The configuration therefore may need to be modified to allow for faster mass transfer into the organic acceptor phase. This could be achieved by using hollow fibres. This will also make it easier to miniaturise the technique. The use of hollow fibre module is attractive in that mass transfer is not limited to only one side as in the present configuration of MMLLE – MIP technique. Therefore faster mass transfer is expected. The enrichment factors are also expected to improve since ratio of acceptor solution to that the sample will be much bigger. The use of hollow fibres may reduce the amount of solvent used than the 2.5 ml used in this case.

The amount of MIP in the acceptor phase also needs to be increased in the present configuration. This will lead to higher extraction efficiency and less target analyte remaining in the bulk organic. Another point worth trying is to see how temperature may

affect the mass transfer. There is generally a great interest in using high temperature (above 25^{o}C) to improve the extraction process. This is because diffusion coefficient of analytes increases with temperature. Increasing temperature may improve the binding of target compounds in the organic solvent.

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APPPENDIX

Figure A1: The enrichments factors of triazines plotted against extraction time after desorption from the MIP in the MMLLE – MIP extraction. These enrichments factors were obtained before concentration of the elution solvent (90 % methanol / 10% water). After concentration of the solvent, the enrichments factors were in the region of 40s.

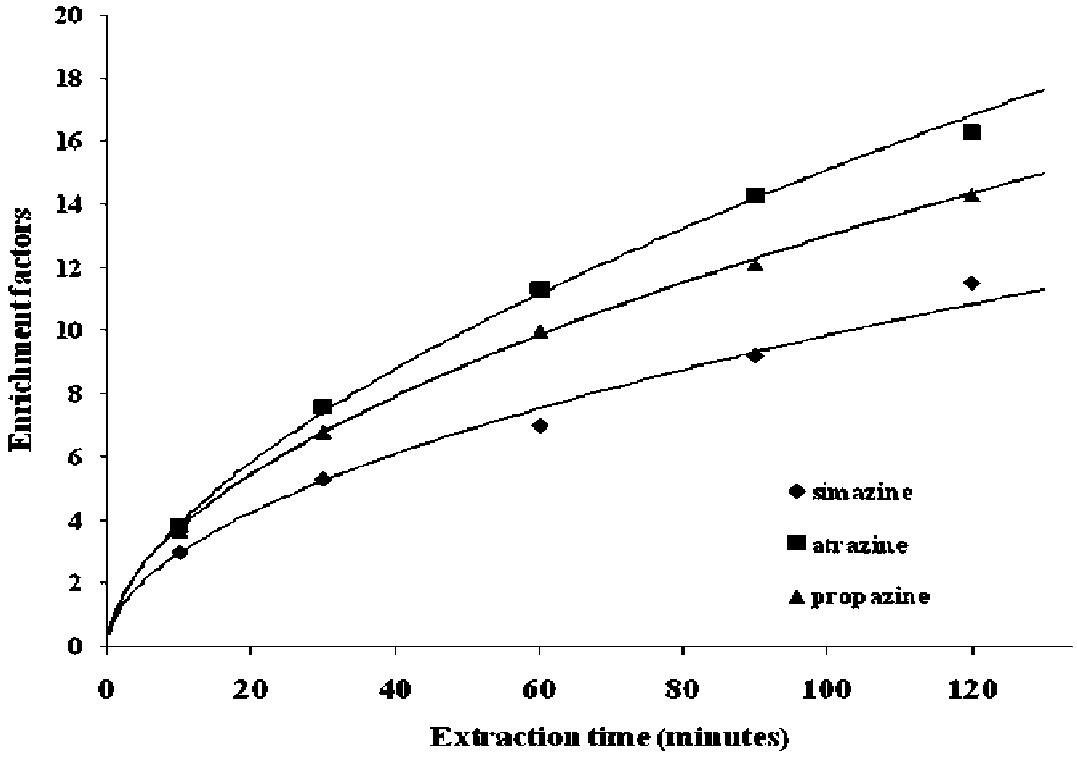


Table A1: Relationship between the permeation constant, Log P and the half life of the triazines in this study.

Compound	MMLLE-MIP permeation constant, k (min^{-1})	Log P	Half life (min)
Simazine	0.016	2.3	44
Atrazine	0.020	2.7	34
Propazine	0.033	2.9	21

Table A2: The effect of varying extracted concentration on the enrichment factors

Concentration (mgl^{-1})	Enrichment Factor (En)		
	Simazine	Atrazine	Propazine
2.5	24	26	23
5	24	27	23
15	28	30	26
30	31	34	32