DEVELOPMENT OF A NOVEL EXTRACTION TECHNIQUE BASED ON COMBINATION OF MOLECULAR IMPRINTED POLYMERS AND LIQUID MEMBRANE FOR BIOMEDICAL AND ENVIRONMENTAL APPLICATIONS

By

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WITS University, Johannesburg, 2007

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)

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ABSTRACT

In this study a novel technique for extraction of biological and environmental samples was investigated. The developed technique is based on the combination of liquid membrane and molecular imprinted polymers technologies referred to as liquid membrane-molecular imprinting polymers extraction technique. As a model compound, 17β-estradiol was chosen, a hormone known to produce adverse effect in wild life and humans. A precipitation and bulk polymerization methods which produces easily, cleanly and in good yield polymers was used for the synthesis of the molecularly imprinted polymers. The model compound was extracted from aqueous sample through the hydrophobic porous membrane which was impregnated with toluene which also formed part of the acceptor solution. In the acceptor phase, the compound was re-extracted onto MIP beads which were also part of the organic phase. The selectivity of the new technique was demonstrated by extracting river water, waste water and fruit sample. In all different samples extracted clean chromatograms were obtained. The new technique therefore combines extraction and clean-up in one step. The enrichment factors were low and around one but can still be improved.

With ever lasting loving memories of my mother

Makwarela Shonisani Nemulenzi (1935-1990)

"Always in our thoughts"

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I would also like to dedicate this work to my son **Zwavhudi**, my daughter **Rolivhuwa**, my husband **Eric** and the rest of my family members for being patient and taking care of my children while I was furthering my studies. I love you all.

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LIST OF ABBREVIATIONS

LM	**Liquid membrane**
MIP	**Molecular Imprinted Polymers**
DH_2O	**Deionised water**
LC-MS	**Liquid chromatography-mass spectrometry**
HPLC	**High performance liquid chromatography**
GC-MS	**Gas chromatography-mass spectrometry**
TMS	**Trimethylsilyl**
PFD	**Pentafluorobenzyl**
NCI	**Negative ion chemical ionization**
E2	**17β-estradiol**

CHAPTER 1: INTRODUCTION

1.1. Molecular Imprinting Technology

The technique of molecular imprinting, introduced in 1972 by Wulff and Sarha, and expanded by the work of the group of Mosbach in 1980s, has shown to be capable of producing materials with "antibody-like" materials in selectivity (Xu et al, 2004). Molecular imprinting is a way of producing materials which possess high selectivity and affinity for the target molecule. Molecular interaction or bonds involved in producing the imprint may be relatively weak hydrogen bonds, ionic attractions, or hydrophobic interactions; or they may involve relatively strong, cleavable bonds such as esters of carboxylic or boronic acids, or ketals or amines. Molecularly imprinted polymers with non-covalent interaction have increasingly been developed as mimic of natural molecular receptors. Basically, molecular imprinted polymers (MIPs) are extensively cross-linked polymers containing specific recognition sites with a predetermined selectivity for compound of interest (Ye and Mosbach, 2001).

The outstanding advantages of MIPs include their physical robustness, high strength, resistance to elevated temperature and pressures, and inert to acids, bases, metal ions and organic solvents as well as the low cost and ease for preparation (Ramström, 1996). To prepare MIPs, the following important ingredients are required: template, cross- linker monomer, the initiator and choice of the solvent (Ramström, 1996).

1.2 Different polymerization methods

Different uses and potential application of the MIPs demand different properties from the polymers. In response to this demand, different methods to produce imprinted polymers have been developed. So, far MIPs have been prepared by bulk, suspension, two-step swelling, precipitation and emulsion core-shell polymerization. Each of these procedures involves the control of different parameters during the synthesis and produces polymers with different properties. Other methods also employed are film synthesis, aerosol polymerization, and polymerization on silica particles (Pérez-Moral and Mayes, 2003).

1.3 Application of molecular imprinting polymers

Molecular imprinted polymers has been used before in chromatographic separation, in biomimetic sensors, in solid phase extraction for sample enrichment/clean-up, in screening of combinatorial chemical libraries, for in situ product removal during biotransformation process, and down stream product purification. They are furthermore of great potential for drug determination when using; for instance ligand competitive assay (Ye et al, 1998). In fact molecular imprinting polymers can be used to extract trace organics such as drugs, metabolites, endogenous compounds, food additive, pesticides, and other analytes of environmental concern (Ye et al, 1998). The major limitation of MIP sorbents is the loss of selectivity when aqueous sample is directly percolated. This is due to water molecules competing for hydrogen bonding onto MIP sorbents with target compounds and that makes MIP to work best in organic solvents.

1.4 Principle of molecular imprinting technology

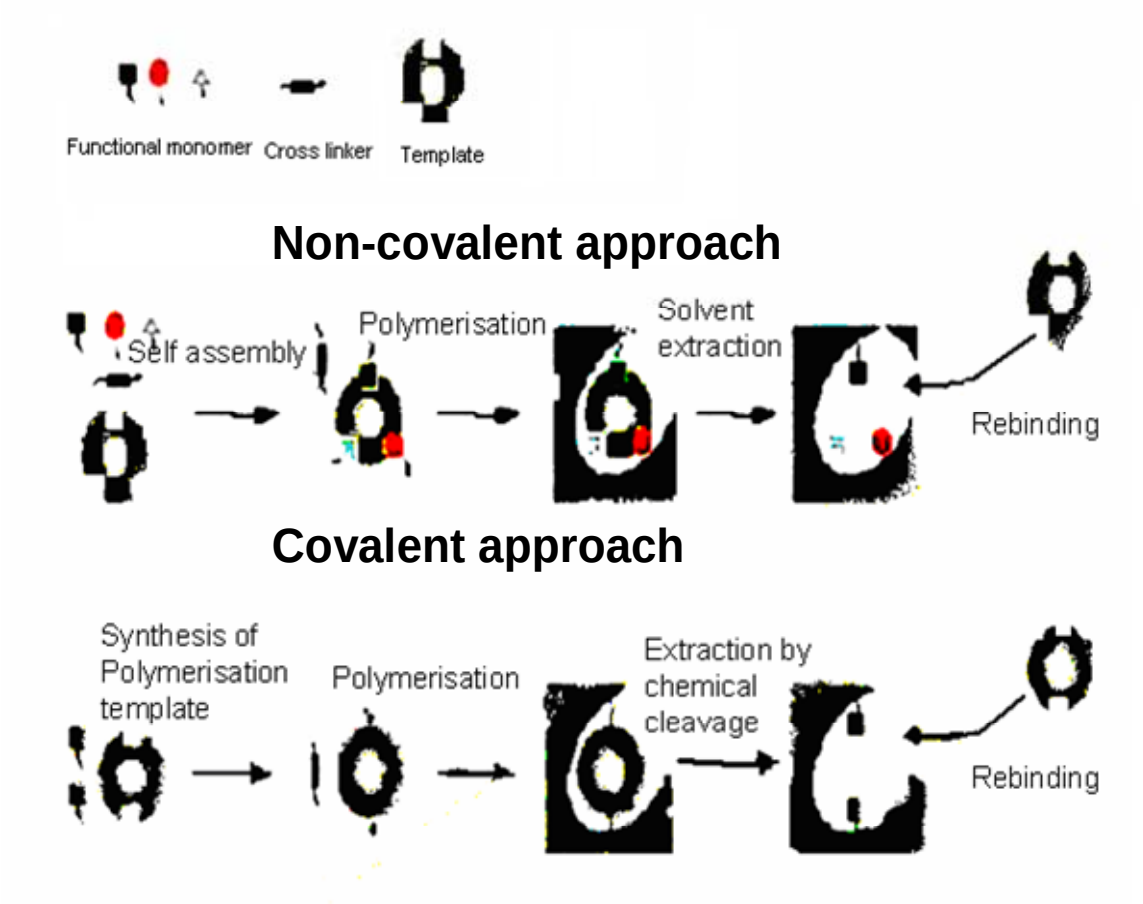

Figure 1. Showing the principles of MIP technology.

Figure 1 show that molecular imprinting polymers can be obtained with covalent or non-covalent approach. Template, functional monomer and the cross linker are mixed forming self-assembly. Polymerization is formed with the cross linker around a template, forming a three dimensional structure. Removal of the template by solvent extraction or chemical cleavage results into binding sites specific to the original template (Ye and Mosbach 2001). In covalent approach, polymerisation produces chemical cleavage with templates which is later re-extracted.

1.5 Fate of 17-β estradiol in the environment

17-β estradiol (E2) is a steroid hormone which also represents the major estrogen in humans and it is suspected of having adverse effects on the endocrine system in wild life and humans. During the excitation of estrogenic receptors, E2 causes the body of wild to change from male to female reproductive form and function (Wei et al, 2005).

Substantial wastes inputs from municipal and agriculture are discharged to many flowing rivers after treatment processes. Natural and synthetic steroid hormones can be carried to agriculture soil through fertilization with municipal biosolids livestock manure, or poultry manure (Jacobsen et al, 2005). The discharge of large volume of wastewater results in the exposure of humans and aquatic organisms to various numbers of wastewater derived contaminants including endocrine disrupting compounds (EDCs). Starting from early 1990s, the researchers began reporting the feminization of the male fish (e.g., the present of egg proteins in their blood). In rivers that receive significant inputs of municipal wastewater effluents, the feminization of male fish was attributable to the present of steroid hormones, such as 17β-estradiol, ethinyl estradiol and estrone (Sedlak, 2005).

The development and implementation of ideal control strategies of E2 is of vital importance. This requires rapid, cost-effective, simple and selective extraction technique for generation of data of E2 in waste water. In many cases these aspects are of in conflict because there is no one simple approach for solving all or even the majority of analytical challenges (Holbrook et al, 2004).

1.6 Liquid membrane extraction as a sample preparation tool

Application of membrane technology in analytical science is currently identified as a very powerful approach because it offers various versions of simple, miniaturized and novel extraction configuration and techniques (Jonsson and Mathiasson, 1999). Liquid membrane was introduced as an alternative separation technique to liquid-liquid extraction technique (Audunsson, 1986).

Liquid membrane composes of two immiscible phases separated by the membrane. One phase is called the donor phase as it contain aqueous solution with the compound of interest which is extracted from it to the acceptor phase. The acceptor phase can be organic or aqueous solution. The compound of interest can be transported across the liquid as a consequence of an existing concentration gradient between the two phases (Ata and Colak, 2005). Membrane selectivity can be fine-tuned by proper choice of the conditions in the different phases (Chimuka et al, 2004). However, in complex samples such as wastewater, sediments, plant extract, the selectivity is insufficient.

1.7 Research objectives

1.7.1 General objectives

- To develop novel miniaturized extraction technique for biomedical and environmental applications in the analytical sciences.

1.7.2 Specific objectives

- To combine the technologies of molecularly imprinted polymers (MIPs) and liquid membrane (LMs).
- To develop a new extraction technique based on the combined LM-MIP technologies, offering unsurpassed selectivity in environmental and biomedical applications.
- To further develop the extraction technique into a miniaturized format, still retaining its simplicity.
- To demonstrate the potential of the new technique using E2 as a model compound.

1.8 Statement of the research

The proposed project carries both academic and industrial significance as it addresses central challenges in present MIP- and LM-technologies. The combination of the two techniques into a working unit has the potential to create synergy effect that may meet these challenges both in terms of aqueous phase performance and analytes selectivity. The LM-MIP techniques is also highly adaptable to versatile and miniaturized production formats, and has the potential to be commercialized.

1.9 Research assumption

This research assumes that the combination of the LM with MIP technology has the potential to produce synergic effect in selectivity and performance.

1.10 Hypothesis of the research

The combination of MIPs and LM will resolve some of the problem faced by these individual techniques in the extraction of environmental and biomedical samples. Extraction will be performed by the miniaturised extraction system that will exhibit high selectivity.

1.20 Justification of the research

Emphasis on the novel extraction techniques for biomedical and environmental applications in the analytical sciences has continued to attract great interest in the scientific community. At first it was directed at developing techniques that are capable of extracting as many compounds as possible with minimal organic solvents for multi-residue analysis. Later, the focus shifted more to techniques that are capable of fast response times and high sample throughput such as immunoassays. Recently, much attention has been concentrated on simple and miniaturized techniques.

Extraction techniques that have been accepted by the scientific community in analytical sciences include solid-phase micro-extraction (SPME) (Beltrans et al, 2000) and stir-bar sorptive extraction (SBSE) techniques (David and Sandra, 2007). However, these novel techniques mainly use extraction sorbents that interact with the analytes through hydrophobic interactions, and this makes them unsuitable for applications that involve complex matrices samples such as wastewater, plant extracts and biological fluids.

In this project, a novel, simple and selective extraction technique that will address the above short comings is presented for the first time. The technique based on the

combination of molecular imprinted polymers (MIPs) and liquid membranes (LMs), taking advantage of both technologies for resulting into selective extraction.

CHAPTER 2: LITERATURE REVIEW

2.1 Sample preparation techniques

2.1.1 Introduction

Major requirements of good sample preparation techniques

Despite the advances in separation and quantitation techniques, typical methods for sample preparation of different environmental samples involve liquid/liquid or liquid/solid extraction with an organic solvent, often followed by clean-up and preconcentration steps. These methods are time-consuming, labor intensive, and costly, depending on the amount of solvent required. A greater concern over the usually toxic solvents discarded and their impact on the environment has led to the development of cleaner extraction methods.

The well known principles of "green chemistry" may be utilized to formulate its main features. The following features are considered to have top priorities:

- Elimination (or, at least, significant reduction of consumption) of reagents, particularly organic solvents, from analytical procedures;
- Reduction of vapor emission and gases, as well as liquid and solid waste generated in analytical laboratories;
- Elimination of reagents displaying high toxicity and/or eco-toxicity from analytical procedures (e.g., substituting benzene with other solvents);

- Reduction of labor and energy consumption in analytical procedures; per single analyte or per whole analytical cycle (Wardencki et al, 2006).

The irony is that analytical methods used to assess the state of environmental pollution which may may also contribute significantly to pollution significant pollution. Sampling, and especially sample preparation, frequently involves generation of large amounts of pollutants. This is why sample preparation techniques that use a small amount of organic solvent, or none at all, have been developed. Usually they are classified according to the extraction phase used; gas, membrane, and sorbent extraction. One important factor that can be considered when choosing for sample preparations methods is to preconcentration the analytes in the sample so as to increase their level/concentration so as to enhance detection. Other important factors are as follows:

- To isolate the analytes from the rest of the sample matrix/components as it is important because sample components can interfere with the analysis.
- To develop a method that is simple and easy to perform
- To be able to extracts as many analytes as possible. This is important because often there is more than one pollutant in the sample. These should be extracted by the same sample preparation method otherwise it can be time consuming.
- To be easy to automate; extract sample unattended. This will allow the person to do other things and precision will also increase.

2.1.2 Different sample preparation technique

2.1.2.1 Liquid-Liquid extraction (LLE) technique

One of the most versatile techniques for the extraction and enrichment of analytes from liquid samples is liquid–liquid extraction (LLE). Compared to the more recent and popular technique of solid-phase extraction (SPE), LLE offers a higher potential for chemically tuning the separation by incorporating different specific reagents, a higher capacity for interfering compounds, and physical separation of the extracted analyte from the extracted sample. However, LLE also has some well-known drawbacks, such as high consumption of solvents, difficulties of automation and on-line connection to analytical instruments, and an often tiresome formation of emulsions (Jönsson and Mathiasson, 1999).

Liquid-liquid extraction, also known as solvent extraction separates compounds on bases of their distribution partition between the solvent systems. It is an extraction of a substance from one liquid phase into another liquid phase. Liquid-liquid extraction is a basic technique in chemical laboratories, where it is done in separating funnels, as well as a common process in chemical industry and ore processing. Liquid extraction is a valuable process in chemical engineering where the separation of one or more of the components from a liquid mixture is required. A flow-based extraction method that operates at a micro-scale to allow continuous liquid/liquid extraction based on the exploitation of surface tension differences between two fluids has also been reported as well as liquid-liquid extraction, the method can additionally be used to separate gas from liquids (Lusiano et al, 2006).

Normally, extracts from samples, whether of biological or environmental origin, contain many diverse compounds included with the possible analytes of interest. To exclude these interfering molecules, a variety of sample clean-up techniques are usually employed. Liquid-liquid extraction is a well-established technique for working up and purifying solutions of fluids and it is also known to be easy to perform. To ensure that the analytes is almost extracted, the same sample can be extracted several times with different portions of organic phase. The continuous-flow operation enables the system to be used for a wide range of scales simply dependent upon how long the system is left running. Typically it takes only tens of seconds from entering the system to have a worked-up fluid stream exiting the system and any settling time that would be required in batch is reduced to a few seconds. Typically between 30 and 60% more effective than the traditional batch process (http://www.answers.com/topic/liquid-liquid-extraction).

2.1.2.2 Solid Phase Micro-extraction (SPME) technique

Solid-phase microextraction (SPME), developed by Pawliszyn and co-worker in 1989, is solvent-free analytical technology, which has the advantage of simplicity, low detection limits and reproducibility (Dong et al, 2006). SPME has gained wide acceptance for the analysis of environmental samples and more recently it has been shown to be useful for many drug analysis applications, coupled to analysis by standard chromatography instruments, (GC, GC–MS, LC, LC–MS, CE) (Namera et al 1996). Sensitivity and precision are generally good or better than standard methods, the methods themselves are simpler, and solvent use is eliminated (Arthur and Pawliszyn, 1990).

In SPME, analytes move from a flowing liquid sample phase to an immobilized or supported liquid or solid-phase, and includes several embodiments. They include mainly open bed extraction concepts such as coated fibres, vessels, agitation mechanism disks, but in-tube approaches are also considered. Some better address issues associated with agitation and others ease of implementing sample introduction to the analytical instrument. It should be noted that solid-phase micro extraction was originally named after the first experiment using an SPME device which involved extraction on solid fused-silica fibres, and later, as a reference to the appearance of the extracting phase, relative to a liquid or gaseous donor phase, even though it is recognized that the extraction phase is not always technically a solid (Arthur and Pawliszyn, 1990).

In the case of fibre solid-phase micro extraction (SPME), analytes from a sample are extracted by a polymer film coated on a fine 1-cm long fused-silica rod (Figure 2). The rod with the polymer film and extracted analytes is then transferred to a hot injector port of a GC or GC–MS, where extracted analytes are thermally desorbed and transported in the carrier gas for standard separation and analysis. For LC applications, analytes are desorbed from the fibre into mobile phase or another desorption solvent, in a small volume desorption interface (Helrich, 1990).

In the technique of in-tube SPME, analytes are desorbed from a polymeric coating inside a capillary, also into mobile phase or a separate desorption phase. In the desorption process, the fibre and/or polymeric extraction phase are cleaned, and rendered ready for another extraction. Methods are typically developed so that extraction and analysis times are similar. In this way and with the use of an SPME autosampler, analysis is continuous,

with the analysis occurring concurrently with the subsequent extraction (Lord and Pawliszyn, 2000).

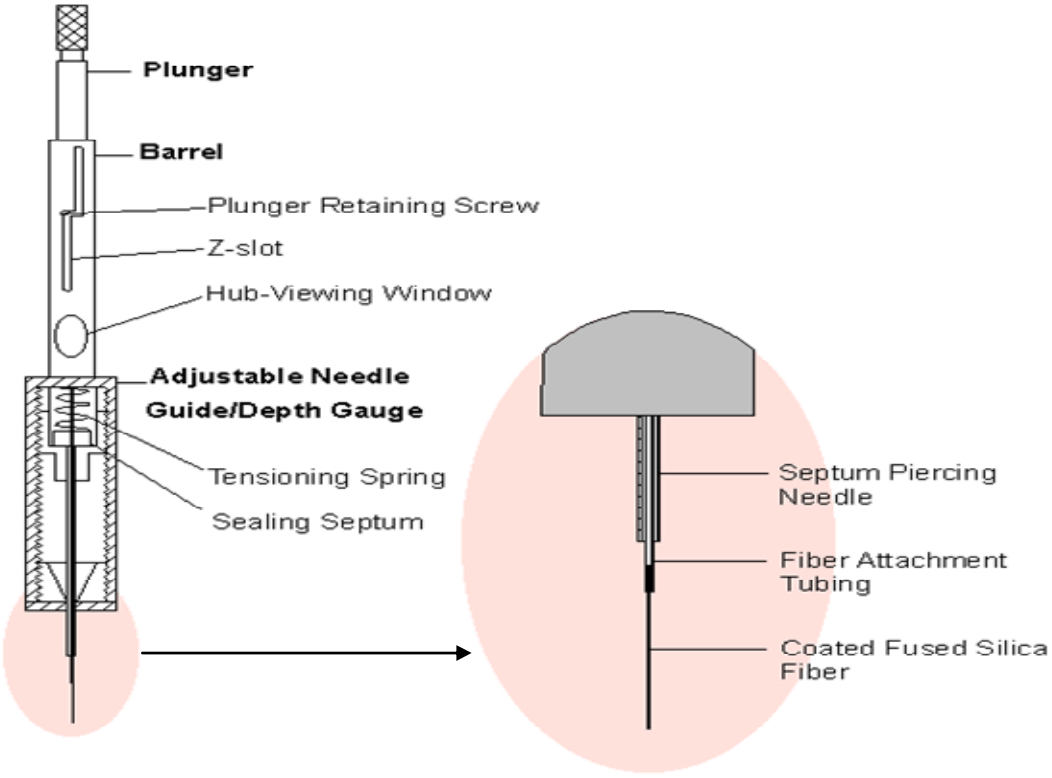

Figure 2. Configurations of solid phase micro-extraction (Lord and Pawliszyn, 2000).

The fiber is mounted in a syringe-like holder called a SPME fiber assembly which protects the fiber during storage and penetration of septa in the sample vial and in the GC injector. This device is operated like an ordinary GC syringe for sampling and injection. The extraction principle can be described as an equilibrium process in which the analyte partitions between the fiber and the aqueous phase (Yang and Xie, 2006).

However, special equipment is usually needed in the SPME procedures and they could be expensive. Moreover, SPME is mostly applied for volatile compounds in liquid matrices.

GC or GC–MS is usually used to determine the targeted compounds. In addition, a fibre blank must be run before every extraction to verify that it is free of contaminants. In fact, SPME has been proven to be an important sample preparation technique applied to forensic specimens and because of the the advantage of being simple, rapid, sensitive and (eliminating) the (disadvantages) of plunging and the use of solvents. The operation is simple, the system can be reused and only small amount of solvent is required. Solid phase micro-extraction (SPME) technique has been well studied and has been applied to some drugs of abuse (Fucci et al, 2003).

Recently, many applications of SPME have been investigated for analysis of semi-volatile and volatile compounds in air, aqueous matrices and in the headspace above dirty aqueous samples, slurries and soils (Psillakis et al, 2000). The technique has been applied for fruit aromas as an alternative sample preparation strategy, to overcome the problems associated with conventional sampling methodologies, such as high costs, time-consumption, and the use of large volumes of organic solvents. In addition, the SPME procedure will more closely reflect the true flavor profile of the fruit pulp than those that might be generated by distillation and solvent extraction processes (Carasek and Pawliszyn, 2006).

2.1.2.3 Solid Phase Extraction extraction

The term "solid-phase" or "sorbent extraction", frequently abbreviated to "SPE", simply implies a physical extraction process involving a liquid and a solid phase. Solid phase extraction also consists of two immiscible phases. However, the extracted phase in this case is a solid material or organic liquid immobilised on the solid support.

This is the most widely used sample preparation method/technique. It has also been the most researched sample preparation technique in the last 10 years. Much research focused on finding different extracting material for example those that can extract very polar compounds/pollutants, materials that can extract on target pollutants (tailor made materials) etc (Poole and Wilson, 2000).

In principle solid phase extraction can be considered as simple liquid chromatography. The sorbent is the stationary phase and mobile phase is the aqueous phase during the extraction step and organic solvent during the elution step. During the extraction step, aqueous sample is passed through the sorbent material. Target analytes are trapped on the sorbent in typical extractions; 50-200 ml of water sample is extracted. The trapped analytes are then eluted by passing a small volume of organic solvent say 3 ml of methanol or acetonitrile. The elution solvent used should be able to remove the analytes (high performance) with low volume (3-5 ml), should be non toxic and should be compatible with instrument to be used for analysis (Poole and Wilson, 2000).

Typical Solid phase extraction sequence consists of four steps:

- Conditioning the sorbent; the surface area of the sorbent is increased say opening up hydrophobic chains in a C_{18} sorbent with methanol.
- Percolating the sample/extraction of the target analytes from the sample by passing in water sample.
- Rinsing and when possible cleaning to remove interfering compounds. Here a small amount of organic solvent is passed but this should not desorb the analytes.

- Desorption and recovery of the analytes from the sorbent using an organic solvent e.g. acetonitrile or methanol.

Various materials of solid phase extraction are available in catridges, column and discs configuration. Catridges have an advantage in that one can have many of them, so that many samples can be extracted at the same time. However, they do not permit online extraction for analysis (Sabik et al, 2000). Disks allow extracting the sample at very high flow rate e.g. 15 ml/min^{-1}. This means short extraction time is taken even for large volume samples giving low determination limits. Discs cannot permit on-line extraction. Solid phase in practice has also come to mean the use of commercial pre-packed columns containing stationary phases related to those used widely in high-performance liquid chromatography (HPLC), that may be adsorbents such as silica gel or FlorisilTM, reversed-phase materials (e.g. with chemically-bonded octadecylsilyl ("ODS" or "C_{18}" groups) or ion-exchange media (e.g. with bonded aminopropyl or phenylsulfonic acid moieties). The packing material is held in a place within a polyemer (usually polypropylene of a serological grade) column by porous frits, also constructed of a polymer material, and the column ends in a Luer tip to facilitate connection to a vacuum manifold, to a needle or to a collection vessel.

Most of the manufacturers will supply copious literature on their products describing the principles of the technique and innumerable applications. One particularly good example of a general handbook is available from Analytichem International (Christie, 1992).

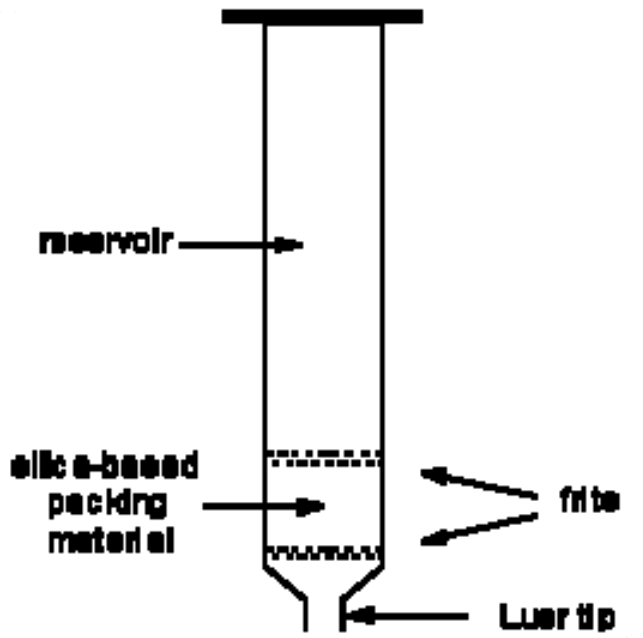

Figure 3. Shows the type of solid-phase extraction column (Christie, 1992).

Break through volume in SPE

Break through volume is the maximum volume of the sample that must be passed through sorbents in SPE. Beyond that volume, the analytes just pass through unretained. Break through volume differs from compounds to compounds and depends mostly on the polarity of compounds and that of the stationary phases. Major causes of the break through volume include: insufficient retention of analytes on the sorbent especially for polar pollutants/compounds and when the capacity of the sorbent is exceeded, that is too large volume has been extracted or sample contain too high concentrations.

The main types of materials used in SPE can be divided into two groups; non selective sorbents/materials and selective sorbents: Non selective sorbents can be used to extract as many pollutants as possible from water but they also extract other unwanted components from the samples. Examples of non selective sorbents are silica materials bonded with C_{18} ad C_8 alkyl chain, graphitized carbon black (charcoal) and polymeric sorbents. Selective sorbents are tailor made to trap only the target pollutants in the sample. Examples of selective sorbents: Immunosorbents (IS): consists of antibodies covalently bonded onto an appropriate material/sorbents and molecularly imprinted polymers which are synthesized materials with antibodies mimics.

The more the compounds are extracted, the wide is the range of their physico-chemical properties, e.g. polar compounds are generally lost during extraction because of low affinity for sorbents. This emphasizes in the careful selection of the sorbent materials due to differences in polarity. Non-polar compounds are efficiently trapped on common C_{18} sorbents but their desorption may be difficulty due to their high retention on the sorbent. Non-polar compounds also adsorb in the tubing and vessel walls because of their high desorption. Very non- polar solvent is therefore used and volume could be more resulting in dilution. Methanol plus methylene chloride combination is often used as elution solvent (Simpson and Dekker, 2000).

To eliminate adsorption phenomena, a low percentage of organic solvent or low amounts of surfactants can be added directly to the sample. Some compounds may exist as ionic forms under normal sample environmental conditions and this may require sample pH adjustments.

One of the advantages of SPE over LLE is that it does not require the use and subsequent disposal of large volumes of organic solvents. Solid phase extraction has a considerable simplicity, reproducibility, reliability and automation capabilities this has made sample preparation using SPE sample preparation to be popular and permanent features of many laboratories (Simpson and Dekker, 2000).

SPE is an extremely efficient method for isolating and concentrating solutes from relatively large volumes of liquid. This technique can be very effective, even when the solutes are present at extremely dilute concentrations (e.g. ppb). Materials extracted in this way can be used for subsequent chromatographic separation, spectroscopic examination, or biological assessment. The apparatus consists of a simple tube, which may be 2-4 mm I.D. and 2-4 cm long and is usually, but not necessarily, made from stainless steel or a suitably inert polymer. The extraction tube is usually packed with an appropriate bonded phase.

SPE is routinely used in many different areas of analytical chemistry. Some of the main fields are environmental and pharmaceutical analysis where cleaning and concentration of the sample are important steps in the analytical protocol. The growth of SPE has largely been at the expense of liquid–liquid extraction (LLE) where the perceived advantages of SPE over LLE are that it consumes less organic solvents and that a wider range of extraction mechanisms can be utilised. Conventionally, solid phase materials have included reversed phase sorbents, such as C_{18}, C_8, normal phases such as silica gel and diol and ion exchange sorbents. For a sorbent to be useful, it must enable selective extractions to be achieved (Olsen et al, 1998).

2.1.2.4 Molecularly Imprinting Polymers

Over the past two decades, enormous activity has taken place in the field of sensor technology. Biosensors, in particular, have attracted considerable attention because of their extraordinary sensitivities and specificities. However, such devices often lack storage and operational stability because they are based on a fragile biological recognition element: an enzyme or antibody. For this reason, biosensors have not become quite the commercial success expected in the early euphoric development phase. An emerging technology called molecular imprinting polymer, however, could provide an alternative. This technique leads to highly stable synthetic polymers that possess selective molecular recognition properties because of recognition sites within the polymer matrix that are complementary to the analyte in the shape and positioning of functional groups (Kriz et al, 1997).

The outstanding advantages of MIPs include their physical robustness, high strength, resistance to elevated temperature and pressures, and inert to acids, bases metal ions an organic solvents as well as the low cost and ease for preparation (Xu et al, 2004). MIPs have high selectivity and affinity constants, comparable with naturally occurring recognition systems such as monoclonal antibodies or receptors, which make them especially suitable as constituents in chemical (biomimetic) sensors for analytical chemistry or even as selective sorbents (Kriz et al, 1997). MIPs have enormous potential applications because of their unparalleled characteristics and have previously been used as chiral separation materials, recognition components in chemical sensors, specific

adsorbents in competitive drug assays, mimics of catalytic antibodies, and solid phase extraction adsorbents for sample preparation (Ye et al, 2000).

The first known examples of molecularly imprinting polymer appear to have been independent of each other, and motivated by two distinctly different goals. The soviet chemist Polyakov was one of many scientists who were involved in investigations of silica for use in chromatography. Polyakov prepared his silica by the acidifications of sodium silicate solution which, after drying of gelatinous silica polymer, afforded a rigid matrix. With the goal of increasing the binding capacity of the silica, the effect on pore structure by the presence of either benzene, toluene or xylene during drying was investigated (Anderson, et al, 1999)

Basically, molecular imprinted polymers are extensively cross-linked polymers containing specific recognition sites with a predetermined selectivity for compound of interests. Molecular imprinted polymers have been used for chromatographic separation, in biomimetic sensors, in solid phase extraction for sample enrichment and clean-up (Ye et al, 1998). Conventionally, MIPs are synthesized by bulk polymerization method in a porogenic solvent creating a block co-polymer. To prepare MIPs, the imprint molecule is dissolved in the acetonitrile porogen or other organic solvent together with functional monomers. The functional monomers will form solution interaction, prearrangement complexes, with the imprint molecule. In that case the bonds may be relatively weak hydrogen bond, potential ionic bond which may be formed between the functional monomers and the imprint molecule. This is followed by addition of crosslinking monomers together with the initiator (Ramström, 1996).

Since free-radical polymerization is inhibited by the present of oxygen, the solution is purged with nitrogen followed by induction of polymerization in the selected condition (Ramström, 1996). After the removal of the print molecule, the polymer will reveal retaining specific binding sites that can selectively rebind the original print molecule (Ye and Mosbach, 2001).

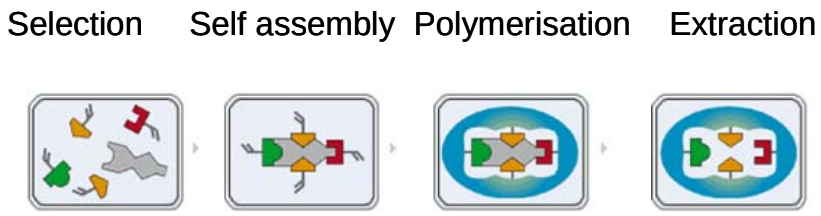

Figure 4. Shows the steps involved in the preparation of MIPs (He et al, 2006).

Methods of MIP preparations

2.1.2.4.1 Emulsion polymerization

This method normally leads to uniform latex particles with diameters smaller than 1 μm. Until now there have been only limited examples of MIP nanobeads that were successfully prepared by simple emulsion polymerisation because the MIP beads generated have narrow size distribution and from which each particle is surrounded by the surfactant ('soap'), the charge on the surfactant repels other particles electrostatically and surfactants and other polymerization adjuvants remains in the polymer and are difficult to remove (Yan and Ramström, 2005). Emulsion polymerization is used to manufacture several commercially important polymers. Many of these polymers are used as solid materials (http://en.wikipedia.org/wiki/Emulsion_polymerization).

2.1.2.4.2 Dispersion polymerisation

This method starts from a homogenous solution containing monomer, initiator and, also often an appropriate stabilizer. The added stabilizer, often a polymer modifier, provides an effective steric barrier to prevent aggregation of the polymer granules. This method is the common one for preparing commodity polymer beads (Yan and Ramström, 2005). The common approach to prepare composite polymer particles is also through multi-step dispersion polymerisation.

2.1.2.4.3 Suspension polymerization

This method has been investigated to the great extent for making molecular imprinting polymer beads, using both aqueous and non-aqueous continuous phases. This method gives larger beads and a broader distribution in particle size, although the latter can be controlled to certain extent by optimizing the reaction conditions (Yan and Ramström, 2005). This technique has been developed for improving binding performance of MIPs and new physical formats of MIPs. This is a simple method to prepare molecularly imprinted polymer beads (Ye et al, 1999). The method turned out to be generally applicable to a broad range of template structures, and purification of the imprinted polymer beads can be easily achieved.

Traditionally, MIPs were synthesized as porous monolith, which after grinding and sieving, gave irregular particles with different sizes in the range of 5–100 μm. Although this method allows easy preparation of small amount of MIPs, it is time-consuming and yields only moderate amount of useful MIPs (yield typically less than 50%).

The irregularity of size and shape of such MIP particles also made sample handling difficult, and chromatography efficiency reduced. For new analytical applications, the irregular particles are inferior to well defined polymer beads, especially in developing MIP-based assays, sensor arrays and separation modules. In addition to improving binding performance of MIPs, new physical formats of MIPs and more efficient synthetic methodologies were important research topics in the past years (Yoshimatsu et al, 2006).

1 Types of interactions in molecular imprinting

MIPs can be divided into two main categories based on the types of interactions between the template molecule and the functional groups, namely non covalent and covalent interactions (Yan and Ramström, 2005).

Covalent was pioneered by Wulff and coworkers (Yan and Ho Row, 2006). This technique involves the formation of covalent bond between the template and the functional monomer in the synthesis of the polymer. During the rebinding, the ligand interacts with the polymer via reversible, labile covalent bonds. Whitcombe and co-workers introduces semi-covalent imprinting which can be looked upon as hybrid approach relying on covalent bonds to first form the template-monomer complex with subsequence rebinding to the polymer occuring via no-covalent interactions (Kirsch and Whitcombe, 2005). Generally, the covalent and the semi-covalent approaches can be successfull in creating well-defined recognition site, but both these techniques suffer from the use of metal-coordination interaction, and therefore these approaches have enjoyed their principal success in rather specific system (Yan and Ramström, 2005).

The introduction of MIP based non-covalent interactions has led to rapid growth in the imprinting field (Vlatakis et al, 1993). Non-covalent interactions are the basis of reversible binding and recognition event in biochemical systems that rely on non-covalent interactions such as salt bridges, hydrogen bonds, and hydrophobic interactions.

This technique is generally regarded as being of more versatile in nature, as much as it can be applied to almost any type of template. The inherent weakness of the interactions makes this approach more difficult to control, often associated with a higher degree of heterogeneity in the binding sites formed (Yan and Ramström, 2005).

The outstanding advantages of MIPs include their physical robustness, storage life, good selectivity, high strength, resistance to elevated temperature, and pressures, and inert to acids, bases metal ions, organic solvents as well as the low cost and ease for preparation (Xu et al, 2004). Disadvantages include, column bleeding, heterogeneous sites and non-polar solvent which is only preferred.

2.1.2.5 Application of Molecularly Imprinting Polymers in solid phase extraction

The potential for MIPs as SPE sorbents was first reported by Sellergren in 1994 a MIP with recognition sites for the drug pentamidin (an antiprotozoal drug) was synthesised and evaluated for on-line SPE. The MIP was prepared using methacrylic acid as monomer and ethylene glycol dimethacrylate as cross-linker. This combination of monomer and crosslinker has subsequently been used for the synthesis of most applications of MIPs for SPE reported to date.

Sellergren achieved selective extractions and concentration of samples when the technique was applied to the analysis of biological fluids (Sellergren, 1998).

A urine sample was spiked with pentamidin and the MIP based extraction resulted in a clean extract and enrichment of the sample to a level where direct detection could be achieved. After demonstration of MIP technology in SPE, it was several more years before the next applications of MIPs in SPE appeared. Probably the most interesting finding in the study was the importance of selecting the correct ionic modifier for the elution step as this greatly affected the selectivity of the extraction.

The use of MIPs for SPE is at an early stage and several successful approaches in bioanalysis and environmental analysis have been reported indicating the potential of the concept. However, a number of problems, particularly with regard to template leaching needed to be solved before the full utilization of MIPs can be realised in the sample preparation arena (Olsen et al, 1998).

2.2 Membrane extractions

2.2.1 Introduction

Liquid membranes consist of a liquid that separates two solutions: the donor and acceptor phases. They can be divided into three groups; i.e. bulk liquid membrane (BLM), emulsion liquid membrane (ELM), and supported liquid membrane (SLM) (Kocherginsky et al, 2006). BLMs consist of two aqueous phases separated by two semi-permeable membranes from a stirrer organic phase. They have the slowest mass transfer rate and have been used mainly in transport mechanism studies. EMLs are formed by

emulsion of three immiscible phases stabilized by surfactants. They have high diffusion rate because of short diffusion path and high membrane-solution contact area (Audunsson, 1986). The problem with EMLs is the formation of stable emulsions and the additional steps needed to recover the analytes (Kralj and Brecevic, 1998). EMLs have been used mainly for industrials applications like the recovery of metal ions from industrial processes. The summary of other liquid membranes is given in Table1.

Table 1. Representing the classification of membrane techniques.

Name	Abbreviation	Type	Phase combinations used Donor/membrane/ acceptor
Dialysis	-	Porous membrane	Aq
Supported-liquid membrane extraction	SLM	Non-porous membrane	Aq/org/aq
Microporous membrane liquid-liquid extraction	MMLLE	Non-porous membrane	Aq/org/org
Semi-permeable membrane devices	SPMDs	Non-porous membrane	Aq/polymer/org
Polymeric membrane extraction	PME	Non-porous membrane	Aq/polymer/aq, Org/polymer/aq, Aq/polymer/org,
Membrane extraction with sorbent interface	MESI	Non-porous membrane	Gas/polymer/gas, Liquid/polymer/gas

Other membrane based extraction techniques

2.2.1.1 Dialysis

In dialysis, solutes diffuse from the aqueous donor side of a porous membrane to the aqueous receiving side as a result of a concentration gradient. Separation between the solutes is obtained as a result of differences in diffusion rates arising from differences in molecular sizes. Dialysis is therefore most effective in removing large molecules like protein from small ones (Van der Merbel and Brinkman, 1993). This technique has few applications for environmental analysis except in biological samples since in the former, both the analytes and the sample matrix compounds are small molecules.

In electrodialysis the electrical potential applied across the separation membrane make sure that properly charged analytes in the feed solution are drawn through the membrane to the receiving side (Jönsson et al, 2003). The advantages found from was that extraction process using electrodialysis can be increased by an external electric field and the techniques were suggested to recover organic acids from their dilute solutions. The distribution coefficient of organic acids was also found to be successfully increased (Pinacci and Radaelli, 2002).

The disadvantage is that sometimes this technique can produce very low electric conductivity of the organic phase and the direct contact of the two phases and the energy consumption can also become high and unstable (Yi et al, 2005).

2.2.1.2 Polymeric membrane extraction (PME)

In this case, instead of a porous membrane, an entirely solid membrane is used to separate the donor and the acceptor solutions. The difference in the solubility and diffusion of various analyte into the polymer is the basis of selectivity. The major advantage is that the solid nature of silicon rubber means that the phase breakthrough is minimized. The major disadvantage is that it does not allow for any room to incorporate other functional group (carrier) that can enhance both the mass transfer and the selectivity of the compounds of interest (Jönsson et al, 2003). Diffusion in solid are also slower compared to liquids so the mass transfer in PME is less compared to liquid membranes.

2.2.1.3 Membrane extraction with sorbent interface (MESI)

The technique is based on membrane extraction into a gas followed by trapping of the analytes on a solid sorbents (cryofocusing) and subsequent thermal desorption into a gas chromatographic system (Pratt and Pawliszyn, 1992). The technique is therefore suitable for volatile organic compounds either in air or aqueous samples. The main draw back of the technique is that it has a narrow application window for environmental analysis; only volatile organic compounds can be extracted (Jönsson et al, 2003).

2.3 Liquid membranes

2.3.1 Supported Liquid Membrane (SLM)

SLM consists of a three phase system which is the aqueous donor phase and aqueous acceptor phase separated by the liquid membrane. In this technique, the sample comes in contact with the organic liquid solvent, immobilised in a membrane (commonly a porous teflon membrane) where the analytes of interest are selectively extracted. On the opposite side of the membrane, a stagnant aqueous acceptor solution traps the inactive (charged/ionized) sample analytes at their optimum pH values. Trapping analytes with stagnant acceptor solutions, results in increased total concentration of the analytes and this leads to efficient enrichment (Audunsson, 1986).

The extraction efficiency of SLM is a function of a number of thermodynamic and kinetic parameters which affect the detection limits, accuracy and precision of quantification of the analytes if not optimized. Therefore, optimization is an important step for an efficient clean-up and pre-concentration of the analyte to be extracted or enriched (Msagati et al, 2005). The composition and nature of the liquid membrane is of critical importance to consider since it can influence the rate of mass transfer, stability and selectivity. The nature and physicochemical properties of the analytes to be enriched, the solubility in water of the membrane solvent, together with the environment in which they are found, provides crucial criteria for the choice of the membrane solvent (Msagati et al, 2005).

Three different physical realisation of SLM modules have generally been reported i.e the flat, spiral and tubular modules (Figure 5). Common configurations of SLM are flat sheet supported liquid membrane (FSSLM) and hollow fiber supported liquid membrane (HFSLM). Small experimental laboratory setup usually consists of a two-compartment cell, separated by a flat membrane. If the SLM is not stable and organic liquid does not stay in the pores, it is possible to use cells with three compartments where two porous supports of same (Wodzki and Sionkowski, 1995) or different (Kislik and Eyal, 1996) nature are used to separate the organic and aqueous phases. The organic solution with the carrier can be stirred or circulated in the middle compartment to decrease mass transfer resistance. PTFE is the one commonly used membrane. There has been many studies conducted using this technique over a wide range of samples (Lee et al, 2007). Also SLM can be modified into a probe that can be used for trace analysis (Cukrowska et al, 2004).

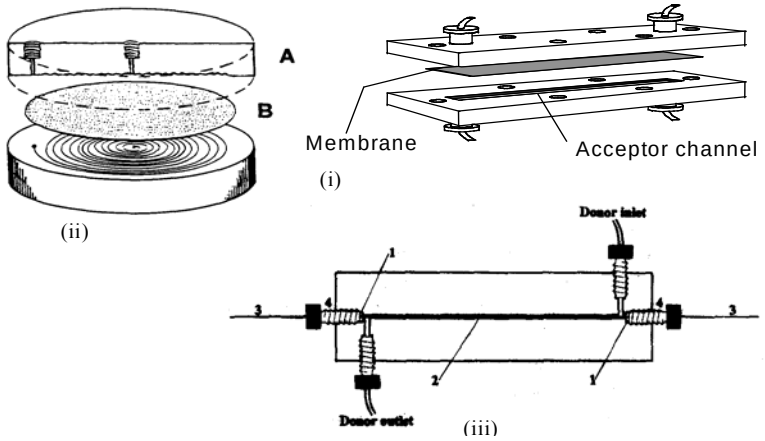

Figure 5. Shows the different types of SLM modules (i) Flat, (ii) spiral and (iii) tubular (Jönsson et al., 2003).

Although SLMs have been widely studied for the separation and concentration of a variety of compounds and present many potential advantages over other separation methods, there have been very few large scale applications of SLM due to insufficient membrane stability. This problem can be due to the loss of the carrier and/or solvent from the membrane, which has an influence on both flux and selectivity. Time after which instability phenomena are observed varies from less than 1 h (Takeuchi et al, 1987) to several months (Danesi, 1984; 1987). The major degradation mechanisms are:

- Progressive wetting of the pores in the membrane support by the aqueous phase
- Pressure difference over the membrane
- Mutual solubility of species from the aqueous phase and liquid membrane phase
- Emulsion formation in the liquid membrane phase
- Blockage of membrane pores by precipitation of a carrier complex at the surface

SLM stability can be affected by the type of polymeric support and its pore radius organic solvent used in the liquid membrane, interfacial tension between the aqueous and membrane phase, flow velocity of the aqueous phases, and method of preparation (Chiarizia, 1991; Yang and Fane, 1997).

The technique has been used as a sample preparation alternative to many others in the extraction of different compounds in avariety of matrices (Jönsson and Mathiasson, 1999a, b). Audunson (1986, 1988) first reported the use of SLM in the determination of amines and after that the technique has been used in many other applications such as enrichment of metals in natural waters (Djane etal, 1997; Ndung'u et al, 1998, 1999).

The technique has also been used in monitoring of a variety of classes of veterinary drugs in biological matrices (Msagati and Nindi, 2001, 2004) and in the enrichment of herbicides in natural water samples (Chimuka et al, 1997).

2.3.1.1 Microporous membrane liquid-liquid extraction (MMLLE)

Another variation of SLM is termed microporous membrane liquid–liquid extraction (MMLLE), a two-phase extraction, where an aqueous phase and an organic phase are separated by a hydrophobic membrane in a flow system similar to that used for SLM. By means of this technique, classical LLE methods can be set up in an alternative manner from conventional extraction-funnel operation or to segmented-flow systems. This approach has demonstrated possibilities for automation, reduced solvent consumption, no emulsion formation, as well as a greatly reduced need for manual labor (Shen et al, 1998).

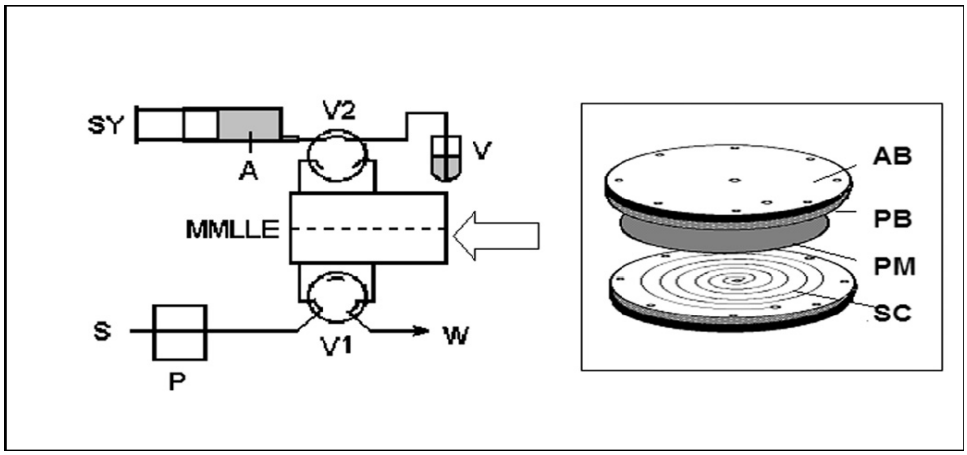

Figure 6. Shows schematic representation setup of MMLLE. P, pump;V1, V2, valves;; SY, glass syringe; V, vial; S, sample; A, accepter (hexane); W, waste; AB, aluminum backer; PB, PTFE block; PM, PTFE membrane; SC, spiral channel (Liu et al, 2003).

The MMLLE technique can be operated either with a stagnant and a flowing acceptor (Sandahl et al, 2000). MMLLE is conceptually similar to continuous dialysis, are also valid for that technique under the assumption of equilibrium between the phases. By using an acceptor flow in the opposite direction to the donor flow, the concentration gradient between the two phases will be larger and the mass transfer will be somewhat more efficient (Bernhardsson et al, 1985). This situation is mathematically far more complex than the parallel-flow assumption, as it is not possible to assume equilibrium anywhere in the cell. For the corresponding problem in dialysis, numerical solutions to the appropriate partial differential equation system have been derived (Bernhardsson et al, 1985).

Compared with SLM, MMLLE has the following characteristics:

- It is applicable to hydrophobic, preferably uncharged compounds, i.e., those that can not be extracted with SLM.
- The maximum concentration enrichment possible is limited by the partition coefficient whereas in SLM it is dependent on the degree of trapping.
- The extract ends up in the organic solvent, not in water. Thus MMLLE is more easily interfaced to gas chromatography and normal-phase HPLC than is SLM, which is most compatible with reversed-phase HPLC.

The hardware is identical or similar, so the possibilities for automation should be similar, considering the point above (Jönsson and Mathiasson, 1999).

2.4 Determination techniques

2.4.1 Liquid chromatography

Liquid chromatography (LC) is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, the different solutes will interact with the other phase to differing degrees due to differences in adsorption, ion-exchange, partitioning, or size. These differences allow the mixture components to be separated from each other by using these differences to determine the transit time of the solutes through a column (Schoeff and Williams, 1993). This technique is widely used for preparative chemistry and biochemistry, in which milligrams to grams of material are isolated (Harris et al, 1997). Liquid chromatography uses liquids as a mobile phase, the stationary phase used are almost exclusively of the octadecylsilyl ("ODS") type, with an octyl phase being recommended occasionally as an alternative. The mobile phase is either acetonitrile (mainly) or methanol containing some water (Faust, 1992).

High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column. A schematic diagram of a typical HPLC is shown in figure 7. The different components 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. 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).

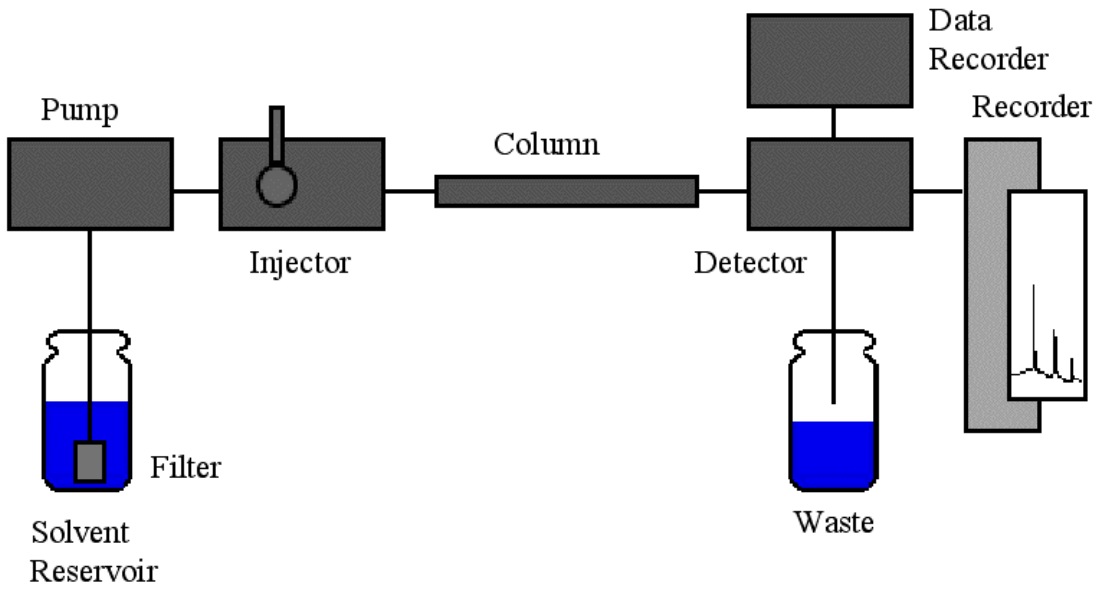

Figure7. High-performance liquid chromatography (Pieper and Rutledge, 1989).

2.4.1.1 Ultra violet detector

The most common detector for HPLC is the UV-Visible spectroscopic detector, which is both sensitive and linear (Smith et al, 2005). UV absorption detectors respond to those substances that absorb light in the range 180 to 350 nm. Many (but not all) substances absorb light in this wavelength range, including those substances having one or more double bonds (p electrons) and substances having unshared (unbonded) electrons, *e.g.* all olefins, all aromatics and compounds, for example, containing > C = O , > C = S , – N = N – groups. The sensor of a UV detector consists of a short cylindrical cell having a capacity between 1 µl and 10 µl through which passes the column eluent. UV light is arranged to pass through the cell and fall on a photo–electric cell (or array). The output from the photocell passes to a modifying amplifier and then to a recorder or data acquisition system. The relationship between the intensity of UV light transmitted through a cell (I_T) and the concentration of solute contained by it (c) is given by Beer's Law which is represented as follows:

A=ebc

Where A is absorbance (no units, since $A = log_{10} P_0 / P$), e is the molar absorbtivity with units of L/mol cm^{-1} , b is the path length of the sample - that is, the path length of the cuvette in which the sample is contained and c is the concentration of the compound in solution, expressed in mol/L (http://www.chromatography-online.org/HPLC-Detectors/UV/rs37.html).

High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection has been also used to determine relatively high concentration (~μg/L) organic compounds; e.g. PCBs and estradiols (E2) from water supplies (Matsumoto et al, 2002).

2.4.1.3 High Performance Liquid Chromatography coupled with a Mass Spectrometry detector via an ElectroSpray Interface

The MS continuously monitors the HPLC solvent flow. When a compound of interest (analyte) elutes from the separations module, the MS first vaporizes the HPLC solvent to remove it, and then it ionizes the compound. The MS then electronically propels the ion through an extraction cone, then into a molecular-mass sorter and finally, into a particle counter. A computer monitors the particle count for a range of masses and ion charges. The values generated provide a mass spectrum that shows information about an analyte's identity as well as its concentration in the sample.

High Performance Liquid Chromatography coupled with a Mass Spectrometry detector via an ElectroSpray Interface (HPLC–ESI-MS) was also found to be useful for the analysis of EDCs e.g. E2 from marine sediments and biota samples sewage effluents and surface waters (Pojana, 2007).

2.4.2 Gas chromatography

In gas chromatography, gaseous analytes is 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, but sometimes it can be a solid analytes either gas or

volatile liquid (Harries, 1987). The schematic diagram of gas chromatograph is shown in Figure 8.

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 nonvolatile 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).

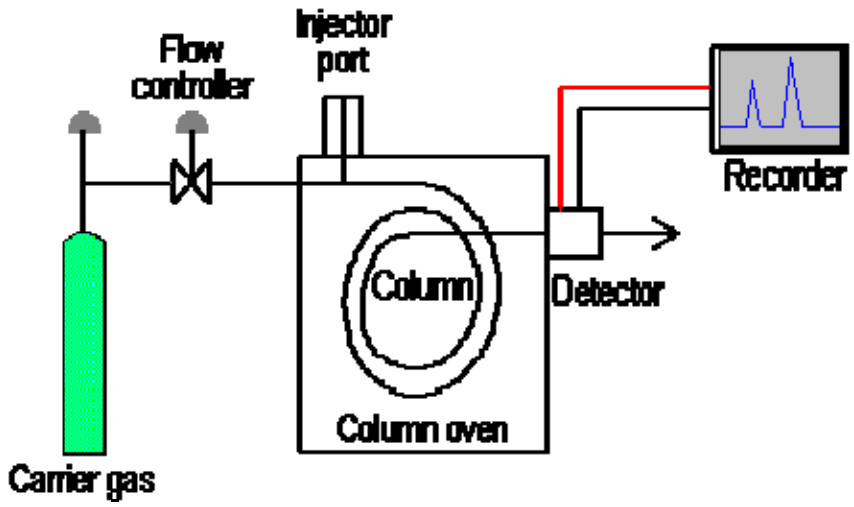

Figure 8. Shows a schematic diagram of gas chromatograph (McCarthy, 2001).

2.4.2.1 Gas chromatography-mass spectrometry

The GC-MS is composed of two major building blocks: gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column and depending on the column's dimensions (length, diameter, film thickness). 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 chromatogram and then that allows the mass spectrometer down to capture, 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 ration.

The analytical determination of E2 from surface and wastewater commonly can be performed using gas chromatography-mass spectrometry (GC-MS or MS-MS) (Yoon et al, 2003). GC-MS can be used to extract E2 from a surface water sample, it analysis by using *N*-methyl-*N*-(*tert.*-butyldimethyltrifluoro-acetamide (MTBSTFA) as the derivatization reagent, and the recoveries can be quantitative if the extract volume is 100–200 μl, irrespective of the amount of reagent added (Yoon et al, 2003).

Estrone (E1), 17β-estradiol (E2), ethynylestradiol (EE2) and estriol (E3) can be measured with the detection limits of 0.65, 0.65, 0.65 and 0.60 ng/ml, respectively when using GC coupled with MS. The recovery for river water samples can be in the range of 86.0–105.1% with the RSD of 1.9–5.8%. The method was applied to the analysis of a river water sample and estrone (E1) was determined to be 2.1 ng/l (Matsumoto, 2002).

2.4.2.2 The flame ionisation detector

The flame ionisation detector is used to measure concentrations of hydrocarbons within a sampled gas. The presence of hydrocarbons is detectable by burning the sampled gas in an air-hydrogen flame. Burning just pure hydrogen with air produces only trace amounts of ionisation. The presence of hydrocarbons in the sampled gas, 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).

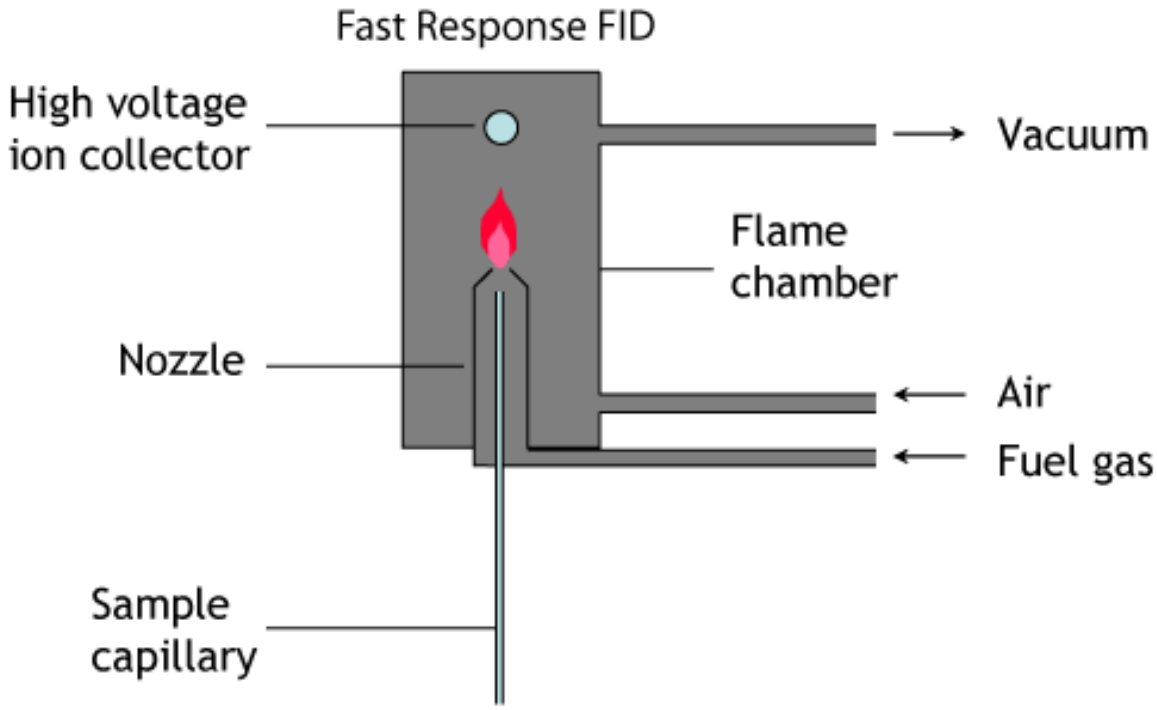

Figure 9. Schematic representation of flame Ionisation Detector (Smith et al, 2005).

2.4.2.3 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 3: EXPERIMENTAL

3.1 Chemicals

97% 17β-estradiol (E2), 2,2′-Azobis(2-isobutyronitrile) (AIBN), 99% methacrylic acid (MAA), trimethylolpropane trimethacrylate (TRIM) and 98% 1,1 Azobis(cyclohexane-carbonitrile) (ACCN) were purchased from Sigma Aldrich (Darmstadt, German). Analytical grade (99.9 %) acetonitrile was from BDH, (London, England). Other solvents used were also of analytical grade.

3.2 Equipments

3.2.1 HPLC and Centrifuge

HPLC used in this project was from SRI (model 210D, LA, California, USA). This instrument composed of variable UV detector from which 220 nm was selected for analysis. A C_{18} column (25 cm x 4 mm, 5 μm) was from Supelco. The instrument was used for the determination of all the analysis. A computer equipped with a Peak Simple version 3.29 chromatographic software was used to process chromatograms.

An MSE, Mistral 1000 bench top centrifuge (Hettich, Germany) was used to sediment the MIPs during washing after synthesis.

3.2.2 Oil bath, ultrasonicator and electron microscope

A silicone heat transfer fluid was bought from Kynethan Business Management (KBM) (Johannesburg, South Africa). The fluid was added as a bath to heat the reaction mixture in the synthesis of molecular imprinting polymer for 17β estradiol.

For shaking or dissolving sample solution during experimentation, a 460 Ultrasonic Elma (Braun, Germany) was used. The physical appearance of MIPs was viewed using scanning electron microscopes JSM-840 (JEOL, JMS-840, Tokyo, Japan).

3.2.3 Membranes and membrane unit

Porous PTFE membrane with polyethylene backing (pore size 0.2 μm, porosity 0.70 μm, total thickness 175 μm of which 115 μm is polyethylene backing, Type FG) was from Millipore (Bedford, MA, USA). The stainless steel extraction unit was constructed by CE engineering cc (Johannesburg, South Africa).

3.3 Preparations of stock solutions

50 mg of 17β-estadiol was dissolved in 50 ml volumetric flask of acetonitrile to make a stock solution of 1000 mg/L. All the standards with the concentration of 0.5, 1, 3, 5 and 7 mg/L were prepared from that stock solution.

3.4 Preparation of mobile phase

Mobile phase was composed of 60 % acetonitrile and 40 % deionised water. The prepared mobile phase was filtered twice and sonicated for 20 minutes in order to get rid of air bubbles which can interfere and disturb the system while running.

3.5 Preparing 0.2M phosphate buffer

The phosphate buffer at pH 7 was prepared by dissolving 20.75 g Na_2HPO_4 and 5.03 g NaH_2PO_4 in 500 ml deionised water.

3.6 Preparation of MIP

The figure below shows the schematic setup for MIPs preparations

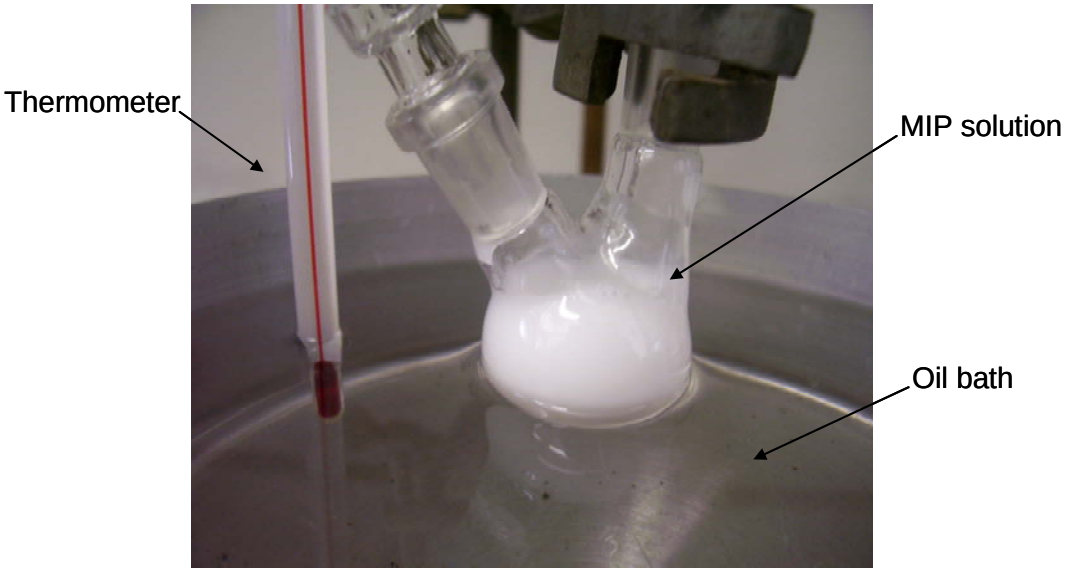

Figure 10. Shows the set up for the preparation of MIPs.

3.6.1 Preparation of MIP by precipitation method

The molecular imprinted microspheres were prepared using precipitation polymerization. The imprint molecule 17β-estadiol, functional monomer MAA, cross linker TRIM and the initiator AIBN were dissolved in acetonitrile followed by sonication for 5 minutes in round bottom flasks. Table 1 summarizes the amounts used in preparation of MIP. The solutions were then purged with nitrogen for 5 minutes and the flasks were sealed under nitrogen. Polymerization was induced by placing the flasks in an oil bath at 60 ˚C.

The polymerization continued overnight. The microspheres obtained were collected by centrifugation using the centrifuging machine. Imprint molecule was extracted by washing three times with 10 mL methanol containing 10% acetic acid, followed by final wash in the same volume of acetone. The repeated centrifugation and decanting steps subsequently removed the print molecule from the polymer. The imprinted polymers were finally dried by leaving the MIPs beads at room temperature. Blank imprinted polymers were prepared and treated in exactly the same way, except the print molecule was excluded during polymerization.

Table 1. Ingredients values used for polymerization preparation.

Ingredients	Molecular weight (g/mol)	Measurments (mg)	Concentration (mmol)	Equivalents
E2	275.39	40	0.145	1
MAA	86.09	60	0.696	5
TRIM	338.40	100	0.296	2
POROGEN	41	8 ml	___	___

3.6.2 Preparation of MIP by bulk polymers

1 mmol of 17 β-estradiol, 8 mmol of monomer (MAA), 25 mmol of cross-linking agent ethylene gylcol dimethacrylate (EDMA) and 50 mg of initiator 2,2-azobis(2,4-dimethylvaleronitrile) (ABDV) were dissolved in 7.5 mL of acetonitrile in an air-tight glass vial. The mixture was then purged with nitrogen for 5 min. The polymerisation was done overnight at 40 °C in a water-bath. The bulk polymers were successively ground in a mortar and crushed with ceramic beads in presence of methanol and washed with 5 rounds of acetonitrile/ acetic acid mixture (4/1) followed by 1 round of acetonitrile and 2 rounds of methanol at 60°C with shaking. The polymers were then dried overnight with a vacuum pump.

3.7 The binding constant of the MIP prepared by bulk method

100 µL of radioactive E2 (0.32 pmol, 30 nCi) in presence of different polymer concentrations ranging from 5 to 40 mg/mL was allowed to equilibrate overnight. The polymer particles were removed from the samples by centrifugation at 13000 rpm for 15 min; 500 µL of the supernatant was added to 4 mL of scintillation cocktail in 20 mL scintillation vials and the radioactivity was measured with a β-radiation counter. Experiments were performed in triplicates.

4.0 Extraction-procedures with LM-MIP technique

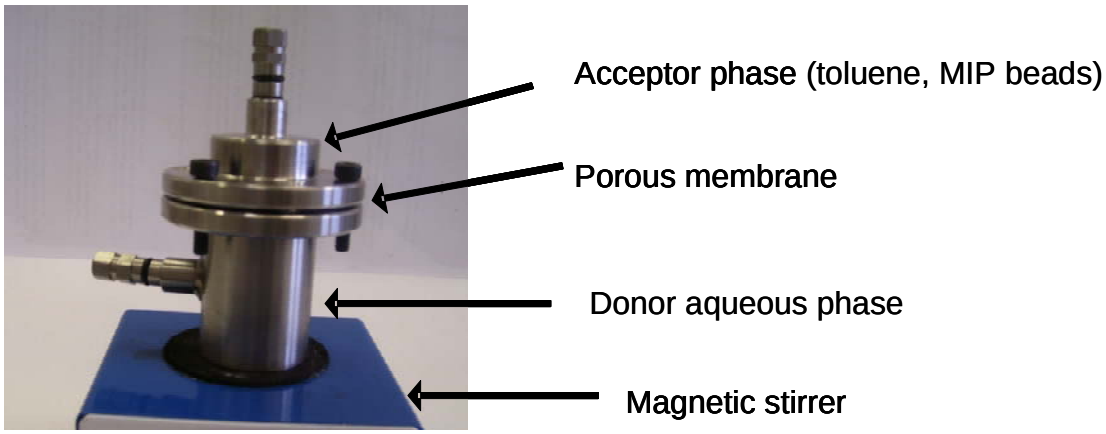

Figure 11. Set-up of LM and MIP extraction technique.

Figure 11 shows the components of the LM-MIP extraction technique. It is composed of two immiscible phases which is the aqueous phase (37 mL) and the organic phase (2.5 mL) separated by porous hydrophobic membrane. The porous membrane was impregnated with the organic acceptor phase, in this case toluene.

7 mg of MIPs beads were incorporated in the acceptor phase. E2 was extracted from aqueous into organic acceptor phase due to its solubility, partition. In the acceptor phase it was re-extracted onto MIP beads. After extraction MIP beads was separated from the organic phase by reducing the volume of the toluene on the membrane and then the membrane with MIP beads was left dry. When toluene evaporated, the beads were then transferred to the vial and 2.5 mL of 10% water in methanol was added for beads to release the bound E2 which was then analysed on HPLC.

Preparation of the extraction unit involved first filling 37 mL of the sample in the lower compartment. The membrane previously soaked for 5 minutes in toluene was placed on top. The upper compartment was then screwed. 2.5 mL of the organic solution followed by 7 mg of MIP beads were then added. The top hole was then closed and the unit was ready for extraction.

4.1 Optimization of LM-MIP technique

Varying the amounts of MIP

This was done by spiking 7 mg/L of E2 in 5 mL of toluene in three test tubes. Then 0.5 mL, 1.5 mL and 7 mg of MIP beads were added respectively. After shaking and leaving the test tubes standing for 30, 60 and 288 minutes, the amount of E2 not bound was determined.

Influence of sonication on E2 rebinding

7 mg of E2 spiked in deionised water was extracted as described in the procedure. After 60 minutes extraction time, the organic solvent and MIP beads were taken and ultra sonicated for 30 minutes before separating the MIP. The experiment was repeated with 60 minutes sonication.

Varying the extraction time

Deionised water was spiked with 7 mg/L of E2 in a 50 mL volumetric flask. The concentration of E2 was determined before and after extraction. Extraction was done for 15 minutes, 30 minutes and 60 minutes. 37 mL of each spiked water was extracted as aqueous donor phase and 2.5 mL of toluene was used as acceptor organic phase with 7 mg of MIP beads. The experiment was repeated twice.

Testing of impurities

To check for any impurities in the prepared MIPs, 5 mL of methanol containing 10 % deionised solution was injected on HPLC and about 7 mg of MIPs in methanol containing 10 % deionised was sonicated and filtered. After filtering the filtrate was injected into the HPLC and both chromatograms were compared for any unwanted peaks. The chromatogram for direct injection of 10% water in methanol served as the control.

Quality assurance

The reproducibility of the HPLC system was determined by injecting 1 mg/L standard solution 10 times. In some cases extractions were done in triplicates to test for the reproducibility of the LM-MIP extraction techniques. The calibration curve was used for evaluation of the unknown sample. Linearity of the calibration curve was evaluated by looking at the correction coefficient. E2 from the samples was identified by comparing its retention time to that of the standard solutions.

4.2 Demonstration of LM-MIP selectivity in various samples

4.2.1 River water, urine and fruit samples

Water sample was collected from the river near Supersport Park Pretoria, Centurion (Gauteng province). It was then spiked with 7 mg/L by taking 350 µl from the 1000 mg/L stock solution into the 50 ml volumetric flask, and the flask was filled up to the mark. 37 mL of this water was then extracted by LM-MIP extraction technique to demonstrate its selectivity against matrices found in river water. Urine sample was donated by one of the postgraduate student. The sample was then spiked the same way as for river water. The LM-MIP extraction technique was applied to urine to check its extraction efficiency and its selectivity to E2 from the rest of the impurities. Each experiment was repeated twice and the conditions of the acceptor phase were as reported earlier. After each extraction, the amount of MIP in the bulk acceptor solution was quantified by letting the solvent evaporate and then exchanged for 2.5 mL of 10% deionised in water. The amount of E2 trapped by MIP beads was desorbed and analysed as before.

60 g of each fruits was crushed in the mortar for homogenization. The homogenate was mixed with 160 ml of methanol in a flask and left overnight. The contents were transferred into a 400 ml beaker and allowed the methanol to evaporate (about 2 days) at room temperature. The sample was filtered after 200 ml of 0.2 M phosphate buffer at pH 7 was added. The fruits in the phosphate buffer were then extracted by the LM-MIP extraction unit.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Synthesis of MIPs

As described in the experimental, the preparation of MIPs requires the template, functional monomer, cross linker, the initiator and suitable solvent. According to other authors, a typical imprinting system consists of a print molecule, at least one type of functional monomer and crosslinker, and a porogenic solvent (Ye and Mosbach, 2001). From the experimental chapter (Table 2) it can be seen that the concentration of functional monomer is more compared to that of the cross linker so that MIPs obtained become very specific to the original print compound than if concentration of the cross linker is more than that of the functional monomer.

During preparation, the shape of the template is the one which will guide the binding of the functional monomer and the binding of the cross-linker will form the double helix structure of the polymer. The initiator will be the one which will initiate the polymerisation by forming the radicals during reaction. According to the ingredients use, it can be seen that about 160 mg of MIPs should be obtained after polymerisation reaction is completed, assuming a 100 % yield.

Table 3. The percent yields of the MIP synthesis.

Mass (mg)	Yield (%)
128.6	80 %
135.8	85 %
87.7	59 %
100.9	63 %
20.2	13 %
Mean	Average value: 60 % (% RSD =47 %)

The percent yield of the MIPs which is shown in Table 3 was prepared using precipitation polymerisation method. The average percentage yield of the synthesised MIPs was found to be 60 %. This gives about 96 mg of MIPs. The morphology is shown in Figure 12B. According to Ye and Mosbach (2001), they found that the ideal way for preparing MIP microspheres is that the reaction conditions are compatible with most imprinting systems and in the presence of excess solvent (typically ≥ 95 % by volume, depending on solubility parameter of the polymer and the solvent). This gives uniform microspheres bearing imprinted binding sites with excellent yields (Greater than of 85 %). To synthesize uniform MIP microspheres, choice of the reaction solvent is critical. In the non-covalent approach, the solvent should be non-interfering to the driving force of forming the print molecule-functional monomer complex. In addition, the solvent should

be miscible with monomers and initiator, but be a non-swelling solvent for the polymer formed.

With acetonitrile as a solvent, uniform MIP microspheres are readily obtained when appropriate amount of print molecule and crosslinker are utilized. The obtained yield was found to be low especially when compared with the 100 % yields of the MIPs which were prepared using the convectional bulk method. In producing conventional imprinted polymer beads, large amount of cross-linking monomer is used with a total monomer concentration of around 20–50 % (v/v) with respect to solvent, which gives higher yields (Ye et al, 2001). The reason for getting low MIPs yieldswith this could be due to manual errors when measuring the amounts of the template and the cross-linker because the acetonitrile (porogen) which produces good polymers was used for polymer preparation. This is supported by relative high standards deviations. The temperature fluctuation from 60°c of the oil bath could also have contributed in getting low amounts of MIPs because the reaction was performed in the hood with the oil bath not covered or insulated.

4.1.2 Bulk polymerisation

About 98 % yield was obtained from MIPs prepared using bulk polymerisation which is higher than what is obtained from precipitation polymerisation. The physical appearance of these polymers is shown in Figure 12A. The morphologies and porosities of the resulting imprinted materials were characterized by scanning electron microscopy analysis, respectively using JSP at 2000x magnification. The figure shows that from bulk polymerisation, bigger particles were prepared. In precipitation polymerisation, smaller particles were formed and that formed clusters or aggregates.

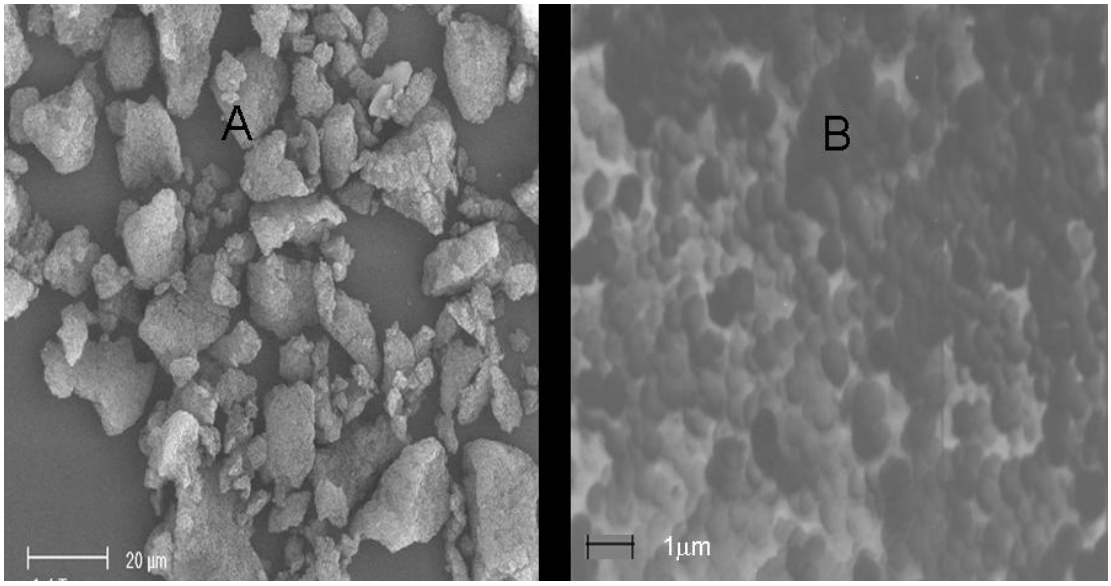

Figure 12 (a) represents the morphology of the MIPs prepared using bulk method and (b) represent the morphology of the MIPs prepared by precipitation method.

Depending on the polymerisation method used, MIPs (Figure 12A), can have various physical configurations. It has been found that the conventional molecularly imprinted polymers or MIPs are irregular in shapes which are combination of macroporous and microporous polymer beads. Bigger MIP particle generally results into slow diffusion of analytes in and out due to large pores compared to imprinted microspheres (Ye et al, 2001). This may be due to the small size of the discrete microspheres and the better accessibility to their specific binding sites.

4.1.3 Binding studies

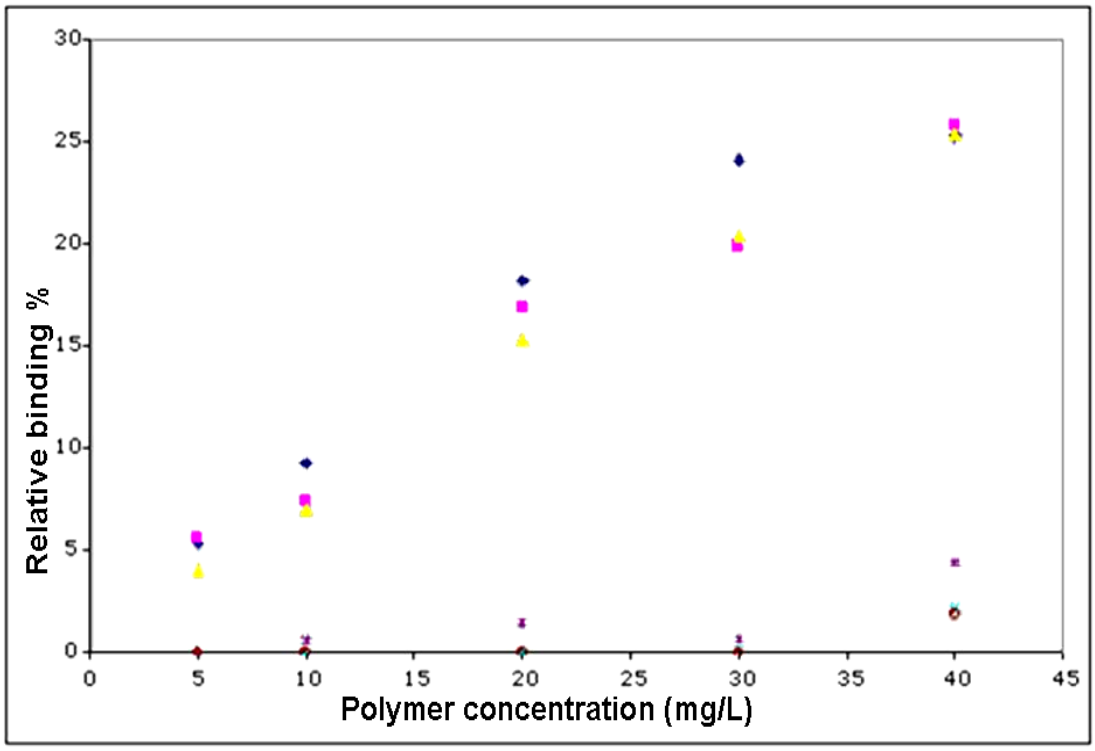

Figure 13. Represents the binding constant of bulk MIPs, results are binding studies of three replicate for MIP and NIP.

The competitive binding analysis was performed to verify the presence of specific binding sites on the molecular imprinted polymer (MIP) and non-imprinted microspheres (NIP). Figure 13 shows that for the MIPs, the binding efficiency increases with the increase in the concentration of MIPs until the binding becomes independent of MIPs concentration.

Moreover, it is hypothesized by Wei and Mizaikoff (2007) that the specific interactions between template and monomers are related to the porosity of the molecular imprinted

polymers. This implies that the amount of binding sites and the pore sized distribution of the molecular imprinted polymer materials are a critical factor in achieving the desired MIP performance in various analytical applications.

4.1.4 Checking for impurities in the prepared MIPs

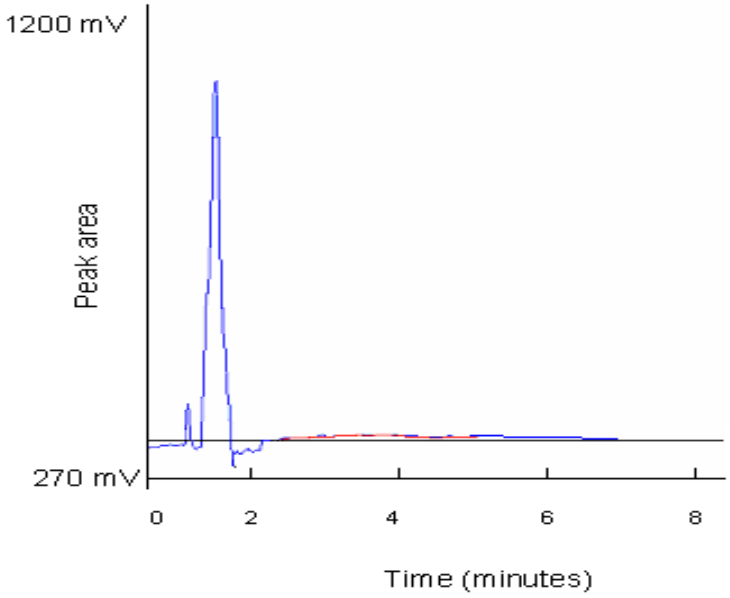

Figure 14. Chromatogram obtained after third MIP wash with 5 mL of 10 % deionised water in methanol.

Template extraction from the prepared MIP is a crucial step as this leaves selective cavities ready for analyte recognition. Methods that have been used by other authors to purify MIPs beads is Soxhlet extraction followed by supercritical carbon dioxide extraction (SFE) with CO_2 at mild conditions (150 bar, 50 °C). At least a removal of >99.7 % of the template was achieved (Bunte et al, 2007). In another study (Bravo et al, 2005), the effectiveness of the extraction was evaluated by using fluorimetric analysis of the washing solutions containing the extracted template (collected after the successive

microwave-assisted extractions). It was also found that successive extractions are therefore unnecessary when compared to the most common extraction methods (incubation and soxhlet). Microwave-assisted extraction reduced extraction time by several hours and in addition, less solvent was used (Bravo et al, 2005). In our studies no impurities were found that could have interfered with analysis of other samples see (Figure13). This procedure which was used for washing MIPs removed most of the unwanted peaks especially around the retention time of 17β-estradiol (8 minutes). This suggests that most of the template extraction was removed.

4.1.5 Quality assurance

Reproducibility

Table 4. Repeated 10 times injections of 1ppm standard solution of E2 in the chromatographic system.

Standard solution injection	Peak area
1	18.03
2	16.84
3	14.76
4	18.17
5	18.66
6	18.11
7	14.44
8	18.19
9	18.39
10	12.17
Mean	16.78 (%RSD=13.2)

The HPLC was tested for reproducibility by injecting 1 ppm of a standard solution 10 times. The standard deviation of 2.22 and RSD of 13.2 % were obtained. It can be said that the analysis was not very reproducible since it was above 5 % RSD. This could be attributed to 17β-estradiol behavior in water standard solution where it is not very soluble

Calibration curve

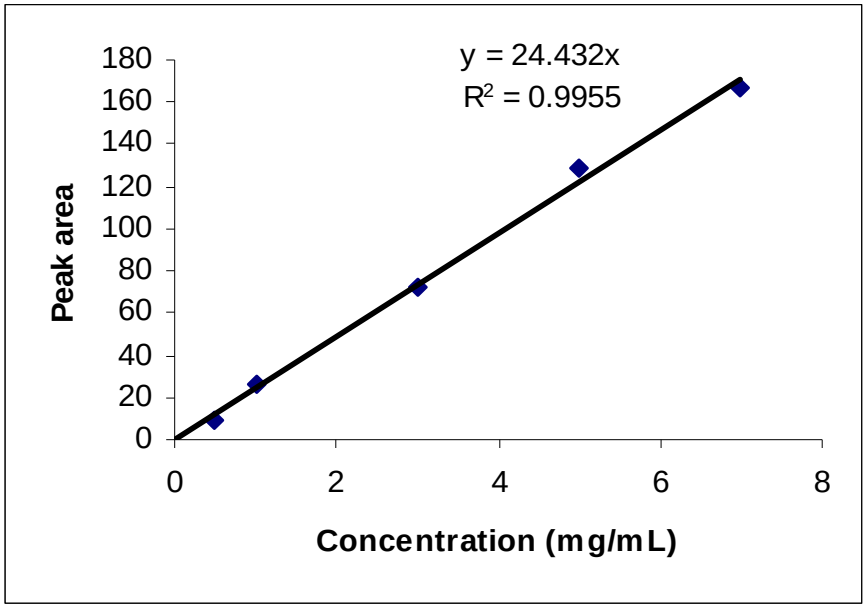

Figure 15. Typical calibration curve obtained from the HPLC system.

A calibration curve was used as a method for determining of the unknown concentration in the extracts. The plot was linear with R^2 values of 0.9955 (Figure 15). This also means that although the HPLC instrument was not very reproducible, it could be used for quantification of concentration of the samples.

4.2 Optimisation experiment of the LM-MIP technique

4.2.1 Variation of the amounts of MIPs

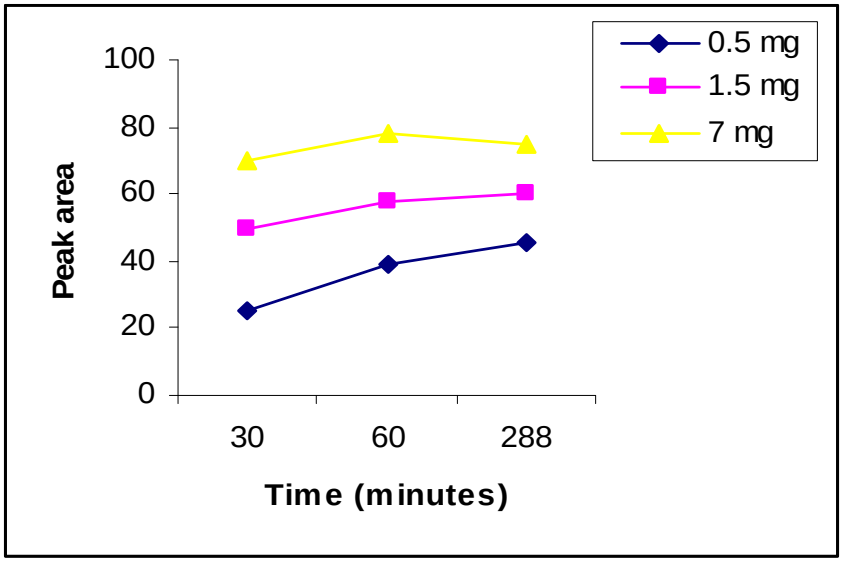

Figure 16. Plot of extraction time vs obtained peak areas with different amounts of MIP.

Figure 16 reveal that binding increases with the increase on the amounts of MIPs and when it is given a longer extraction time. From figure 16 it is clear that much more binding occurred when more amounts of MIP were added and this was more pronounced at lower binding time. The effect of binding/extraction time was more pronounced at 60 minutes. Thereafter the curves tend to move towards reaching a plateau. For the application of the LM-MIP extraction technique, an extraction time of 60 minutes was taken as the optimum. Too long time results into low sample throughput.

From other studies, it has largely been demonstrated that MIPs offer the highest selectivity when samples are dissolved in the solvent used for the MIPs preparation (Pichon, 2007). In our study, toluene was used as a solvent instead of acetonitrile that was

used to prepare MIP. This could have affected the rebinding but could not use acetonitrile because it cannot form a stable liquid membrane in the proposed technique.

4.2.2 Influence of sonication on binding

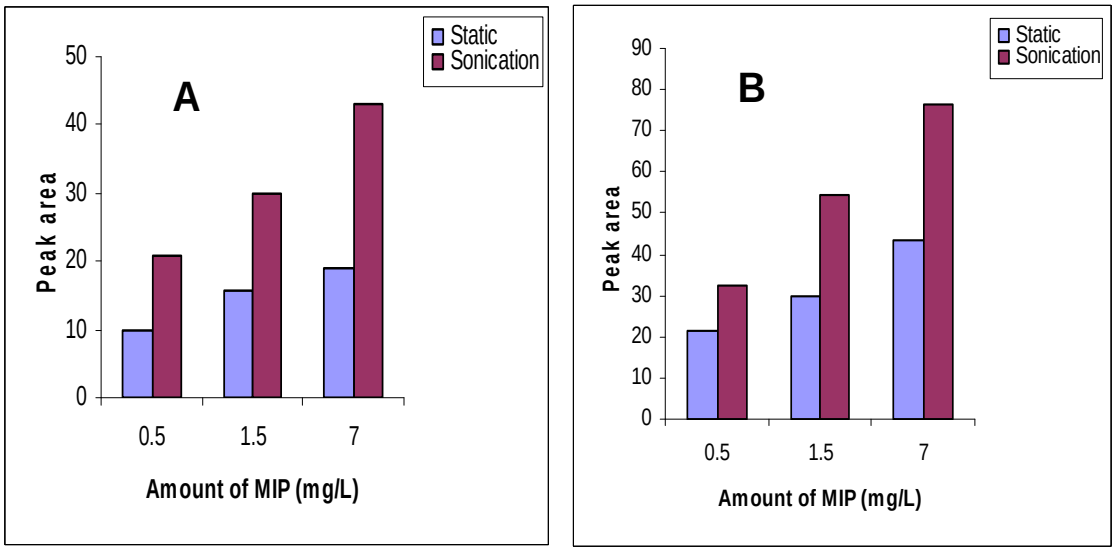

Figure 17. Comparison of rebinding of E2 under static versus sonication conditions. A; 30 minutes and B; 60 minutes binding times.

In order to increase the rebinding of E2 to the MIP beads, sonication was tried with various MIP amounts and sonication time. Figure 17A and B. This was done to the LM-MIP extracts before desorbing the E2 with 5 mL of 10% water with methanol as described in the experimental. The results show that sonication increased the rebinding of E2 to MIP beads. The rebinding was more pronounced at 60 minutes sonication time. Longer waiting time also increased the amount re-bound onto the MIP. The results obtained from both of these experiments suggest that sonication can be used to obtain re-binding of a template from MIPs.

4.2.3 Varying extraction time

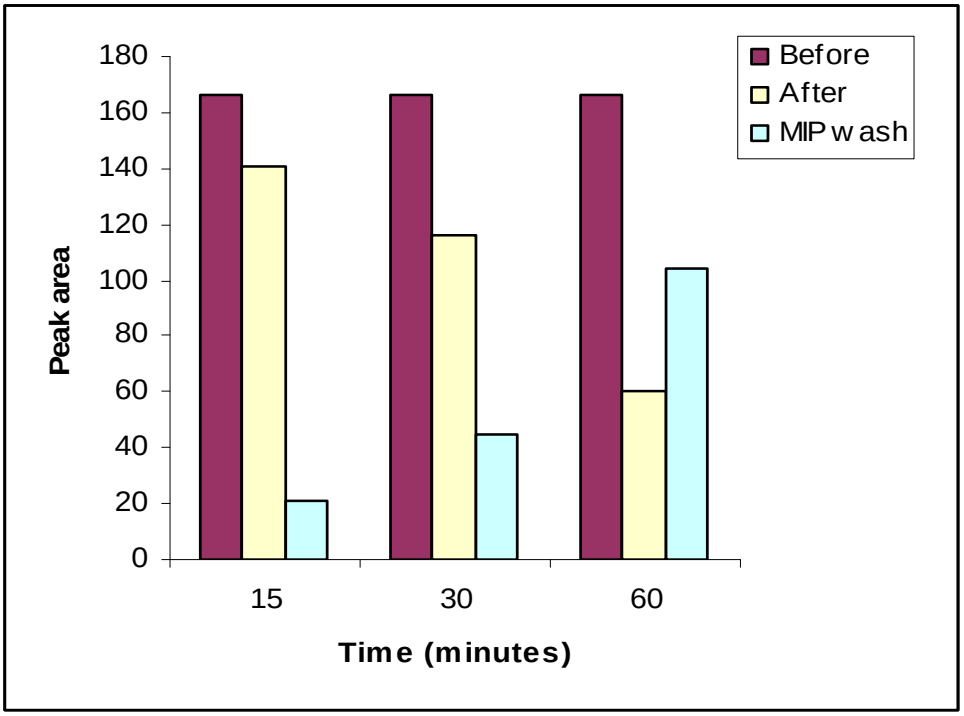

Figure 18. Plot of E2 peak areas at different times in LM-MIP extraction technique.

Figure 18 shows the results of varying the extraction time in LM-MIP extraction unit. The concentration of aqueous solution was analysed before and after extraction to determine the amounts extracted and that remained in the sample. The re-extracted E2 which was retained by MIPs was released by adding 5 mL of 10% water in methanol which was later sonicated for five minutes, filtered and then analysed on HPLC. From the results obtained it was found that about 16 % of E2 was extracted to the organic phase in 15 minutes and 12 % was re-extracted by MIPs. In 30 minutes it was found that MIPs in the acceptor phase retained 27 % of E2 from 31 % that was extracted in the acceptor phase. After 60 minutes about 53 % of E2 was extracted in the acceptor phase and 43 % was MIP bound. When comparing these experiments, it was found that with 15 minutes

extraction time not much of E2 was extracted. More E2 was extracted with a time of 60 minutes. In this case the extraction increased about three times and MIPs. These results agree well with those obtained in figure 18 that E2 rebinding increases with time.

4.3 Demonstration of selectivity

4.3.1 Deionised water

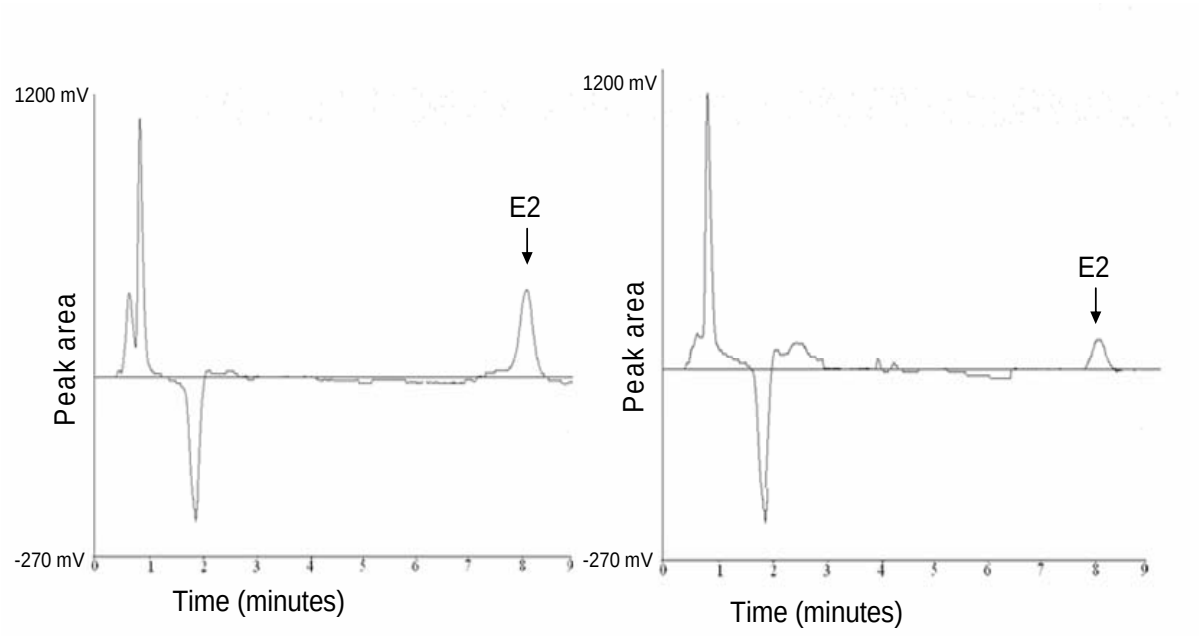

Figure 19. Chromatograms obtained from direct injection of deionised water spiked with 7 mg of E2 (A) and after LM-MIP extraction (B).

It can be seen from figure 19 that the two chromatograms are clean as expected since deionised water has no matrix components. Figure 19B shows low amounts of E2 detected after extraction with LM-MIP extraction technique. This could be due to the rebinding of E2 from the bulk acceptor solution onto the MIP beads.

4.3.2 River water extraction

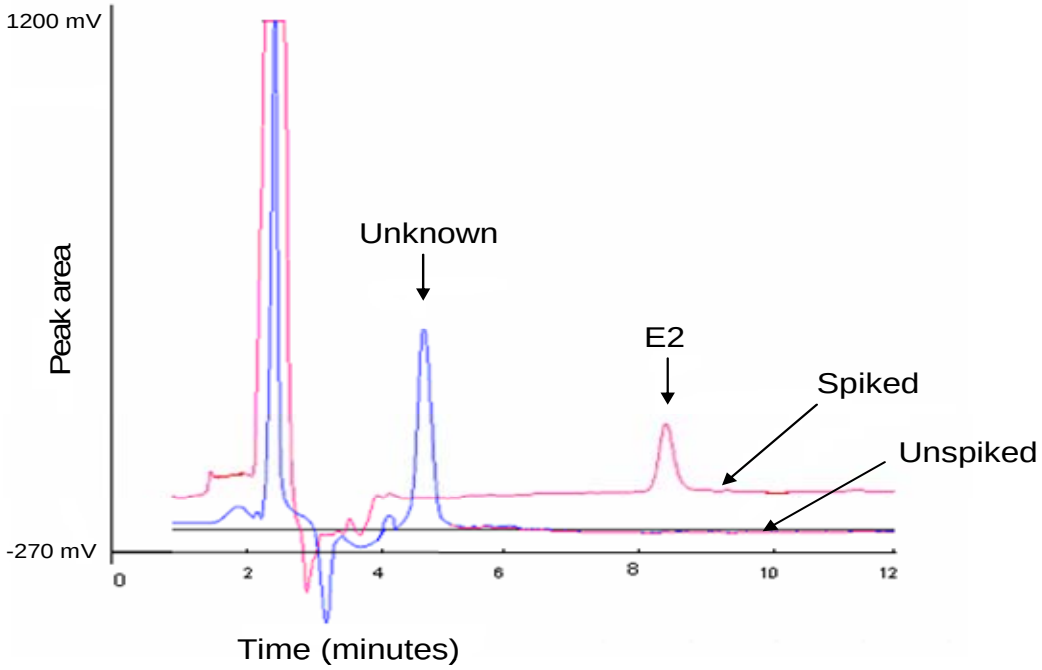

Figure 20. Comparison of the selectivity of LM-MIPs technique in river water Chromatograms were obtain from direct injection of unspiked and after LM-MIP extraction of new water spiked with7 mg of E2.

Figure 20 demonstrates the selectivity of the LM-MIP technique. When river water was injected directly, unknown compound was detected, but after the extraction with the technique it was eliminated. This high lights the novelty of adding MIP beads in the organic acceptor solution. The results are however not surprising since MIPs are known to be specific for the template molecule.

4.3.3 Urine extraction

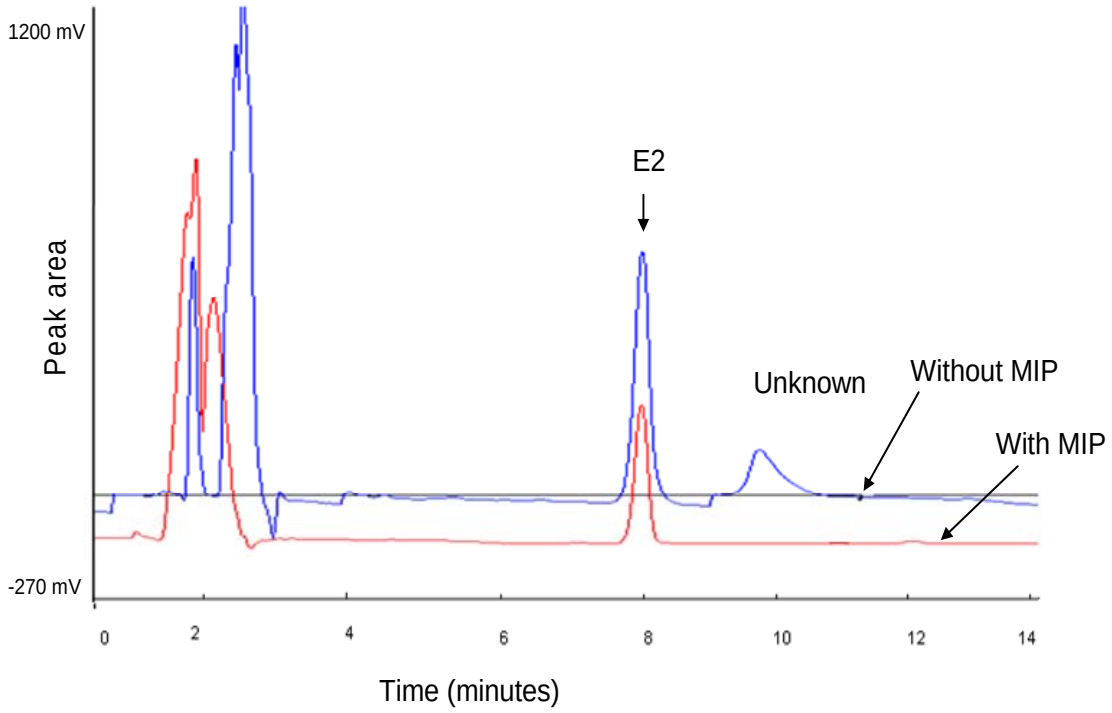

Figure 21. Comparison of selectivity after extraction urine spiked with 7 mg/L E2 with and without MIP beads incorporated in the organic acceptor.

Biologically estrogens are present in biological tissues and fluids mainly as water-soluble conjugates with high polarity, i.e., estrogen sulfates and glucuronides, instead of the free compounds. The three major occurring estrogens in woman are estrone, estriol and estradiol. It was thus important to test the selectivity of the LM-MIP by extracting spiked urine samples. Further prospective epidemiological studies have suggested that additional estrogen, whether from endogenous or exogenous sources, may be associated with the development of breast cancer promotion and tumor growth (Zhang and Henion, 1996).

Figure 20 demonstrates the selectivity obtained. With the organic solvent (toluene) as the extracting acceptor phase, only other unwanted peak is observed in the chromatograms. After the addition of MIP beads, the peak disappears emphasizing the selectivity of the proposed techniques. The presence of the other peak shows that membrane extraction is not very selective. In this case most of other compounds from the sample were extracted too. It should be noted that the liquid membrane with organic acceptor is not very selective since selectivity is based on differences in partition coefficient of the sample components into the organic liquid (Yang et al, 2002). The E2 peak after LM-MIP extraction is smaller due to slow re-binding of E2 onto MIP beads in the organic acceptor solution.

4.3.4 Fruit sample

Different types of fruits sample and other samples were also analysed in order to study the selectivity of LM-MIP extraction technique. Apple, water melon and tea (eleven o'clock, rooibois) were chosen because they represent a wider variety of matrices. E2 is not found in fruits sample but these samples were used to just test the selectivity of the LM-MIP combination.

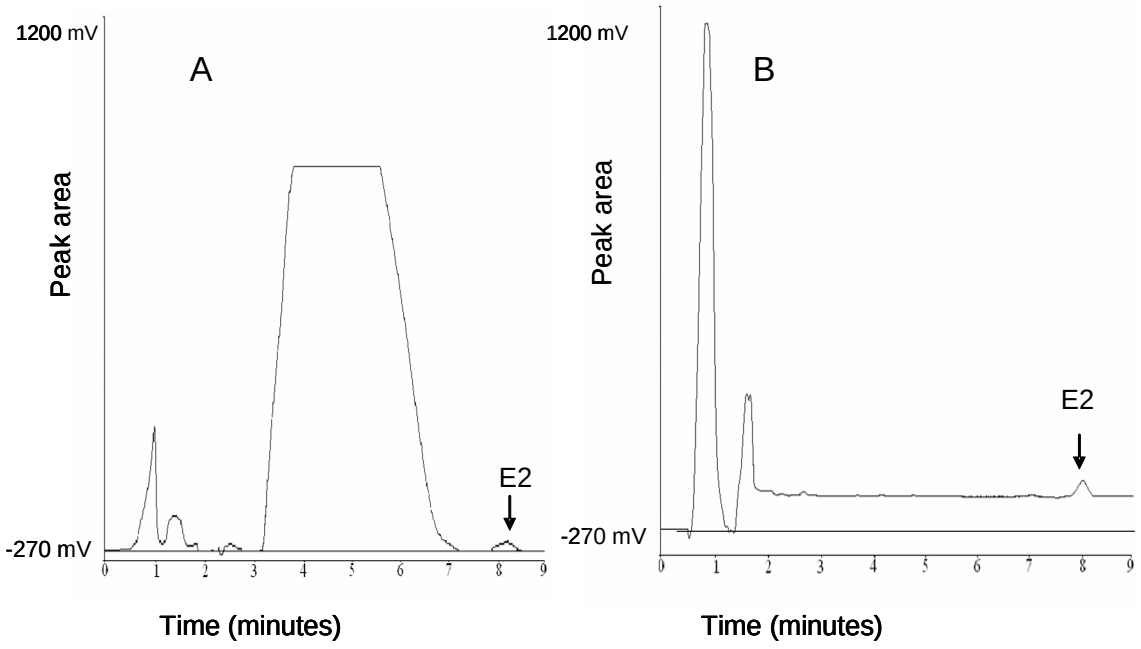

Figure 22. Comparison of selectivity in the apple sample without MIP (a), with MIP (b).

Apple aqueous sample were spiked with 1 mg/L of E2 standard solution. Extraction was done for 60 minutes. Chromatograms show that MIP additions into the organic acceptor phase resulted into selectivity extraction of E2. With the organic liquid only, other unwanted peaks are observed. Khrolenko (2005) also observed that liquid membrane extraction only is not very selective for matrices found in fruits. The addition of MIP made the difference and its selectivity is clear as there was only one peak of E2 (figure 22 b). The selectivity of MIP for estrogens and other compounds also studied and demonstrated by other researchers and good results have been obtained (Bjarnason *et al*., 1999; Meng *et al*, 2005, Le Noir *et al*, 2007).

4.4 Comparison of LLE and LM-MIP extraction technique for the extraction of apple sample

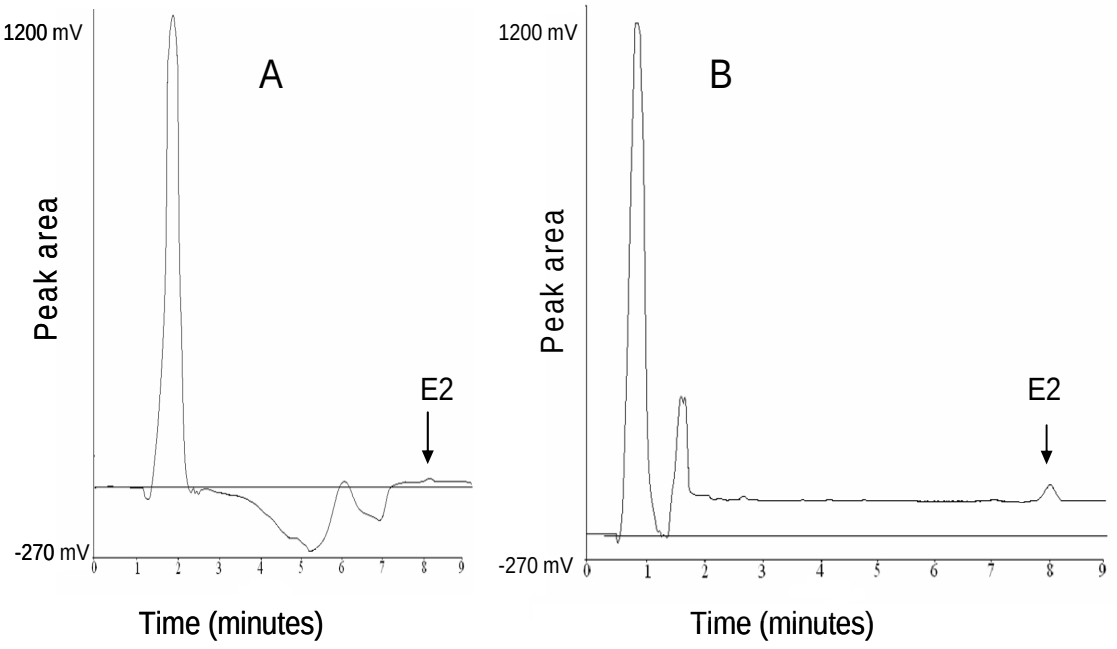

Figure 23. (a) Analysis of apple sample using LLE and Figure (b) Analysis of apple using LM-MIP extraction technique.

From the results showed in the figure 23, it can be seen that with LLE not much of E2 recovered and other compounds were extracted along. In this case liquid-liquid extraction was only performed once not with repeated extraction of the same sample three times which is the standard procedures. It was also stated that When LM-MIPs technique used for extraction of the same sample, more E2 was extracted with very good selectivity.

4.5 Preconcentration factors

Table 5. Summary of the enrichment factors in various samples.

		Spiked (mg/L)	Determined concentration	Enrichment factor
Bound onto MIP beads (acceptor)	Tea	1.0	0.915	0.9
	Apple	1.0	0.969	0.9
	River	7.0	2.9	0.4
	Urine	7.0	3.2	0.4
In hexane (acceptor)	Tea	1.0	1.590	1.6
	Apple	1.0	1.707	1.7
	River water	7.0	5.3	0.7
	Urine	7.0	5.1	0.7

Table 3 gives a summary of enrichment factors obtained in various samples from that found in the organic acceptor phase and from that bound onto MIP beads in the acceptor organic phases. First, results shows that the enrichment factors from the MIP are low in all the samples. This is due to poor re-binding of E2 onto MIP beads. The organic phase contained more E2 than that bound onto MIP. The effects to increase the rebinding with sonication did significantly improve the results neither the addition of more MIP beads. Other method such as ultrasound assisted rebinding will have to be tried in future.

CHAPTER 5: CONCLUSION AND RECOMMENDATION

5.1 Conclusions

A new novel extraction technique for environmental and biological samples was developed. The technique is based on the combination of liquid membrane (LM) and molecular imprinted polymer (MIP) technologies. The developed technique was tested by applying it to river water, urine and apple sample. It was found to be selective to E2 from other compounds in these samples

MIPs for E2 were successfully prepared using precipitation polymerization which produces spherical microspheres and the convectional bulk polymerization which produces block monolith. In evaluation of these polymerization methods it was found that a high yield of spherical microspheres were produced compared to the block monolith. In addition precipitation polymerisation method is simple and it takes less time compared to convectional bulk method as it does not require crushing and sieving of the polymers.

5.2 Recommendations

- The developed LM-MIPs extraction technique though showed high selectivity, the enrichment factors were low. The technique therefore need further optimization to increase the enrichment factors such as trying different organic liquids as acceptor phase and using ultra sound to assist the rebinding of E2.
- Testing the new technique to other organic pollutants such as triazine and polyaromatic hydrocarbons in various complex samples.

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APPENDIX

Appendix 1. Figures of chromatograms for standards solution and those which were determined during extraction experiments.

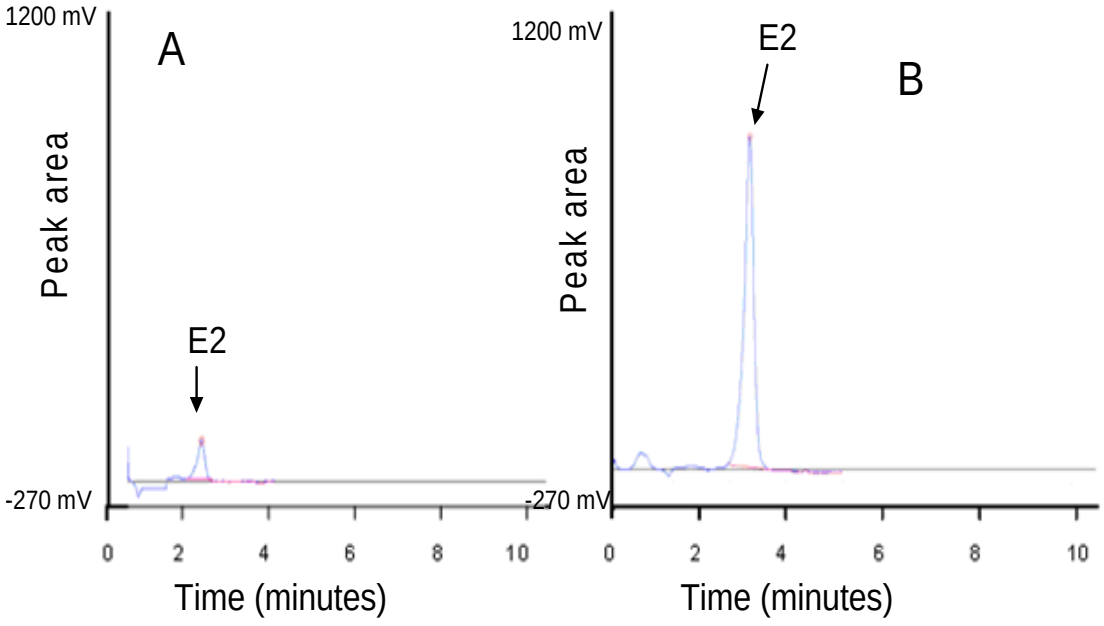

Figure 24. Chromatograms of 0.5 mg/L (A) and 7 mg/L (B) E2 standards solution while using the C_8 column

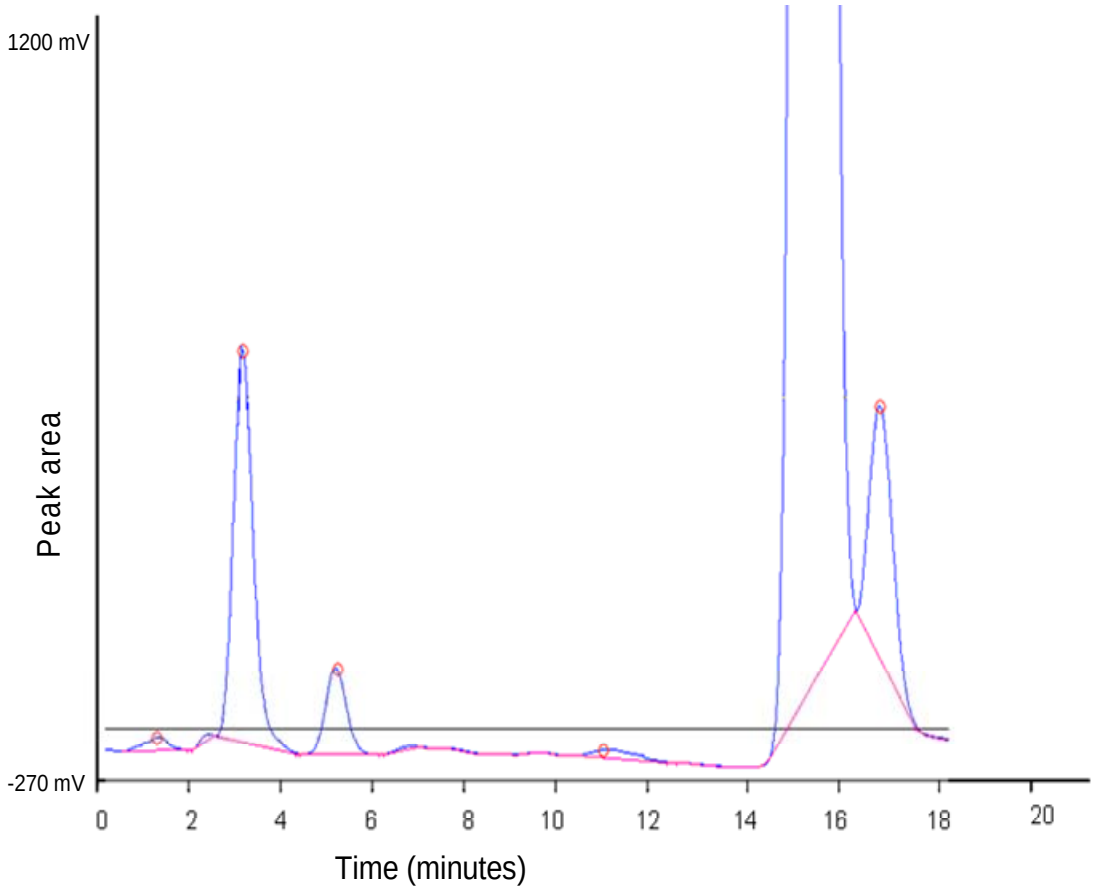

Figure 25. Chromatograms of 5 mg/L using old C_{18} column with mobile phase composition of 70 % acetonitrile and 30 % deionised water.

Appendix 2. Viewing of the polymer using optical microscope

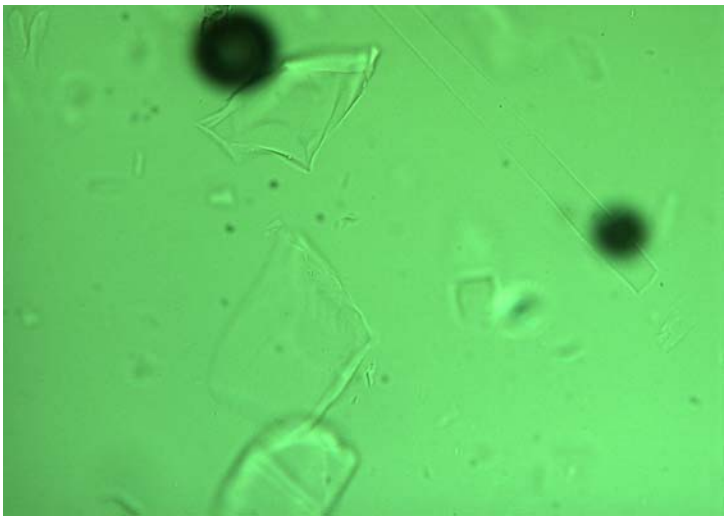

Figure 26. MIPs at 100x

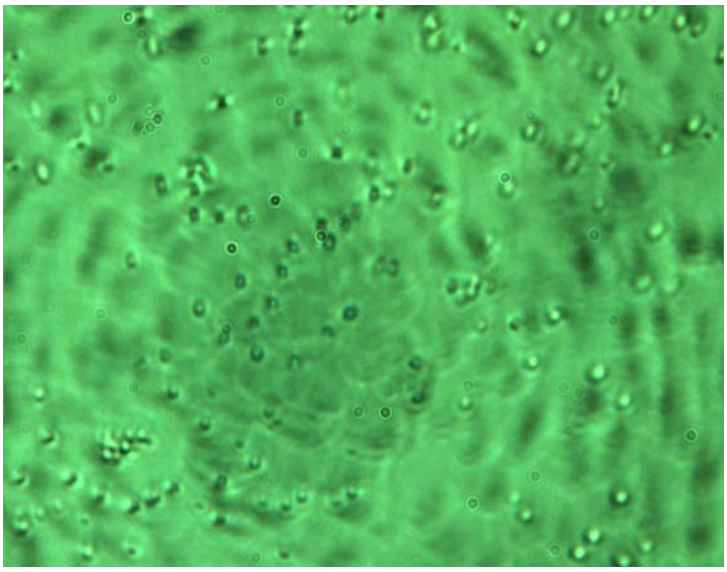

Figure 27. NIP at 100x

Appendix 2. Tables of values determined during extraction of river water and urine samples

Table 6. Values of Plot of extraction time vs obtained peak areas with different amounts of MIP

	0.5 mg	1.5 mg	7 mg
30 minutes	25.4542	49.9512	69.5522
60 minutes	39.0058	57.7252	77.9706
288 minutes	45.1856	59.8624	85.1856

Table 7. Values for varying extraction time

	60 minutes	30 minutes	15 minutes
Before	166.3832	166.3832	166.3832
	166.3832	166.3832	166.3832
	166.3832	166.3832	166.3832
Mean	166.3832	166.3832	166.3832
After	76.7456	64.4608	40.9968
	97.9548	78.0056	45.7528
	79.0080	72.3915	58.7166
Mean	84. 5695	71.6193	48.4887
MIP wash	76.1412	51.7510	36.4780
	100.3008	92.8026	34.0080
	81.5920	61.6486	43.4652
Mean	86.0113	68.7341	37.9737

Table 8. The mean values of varying extraction time

	15 minutes	30 minutes	60 minutes
Before	166.383	166.383	166.383
After	141.155	115.953	77.9028
MIP wash	21.3171	43.4007	80.0113

Table 9. Static versus sonication values in 30 minutes

	0.5 mg	1.5 mg	7 mg
Static	9.9942	15.5232	18.9856
Sonication	20.9706	29.9512	43.1856

Table 10. Static versus sonication values in 60 minutes

	0.5 mg	1.5 mg	7 mg
Static	21.6542	29.8624	43.231
Sonication	32.4556	54.2113	76.6343

Appendix 2. Summary of parameters for LM-MIP technique

UV detection wave length	220 nm
Mobile phase	60 % deionised water and 40 % Acetonitrile
Running time	8 minutes
Retention time of E2	6 minutes
Mass of MIP	7 mg
Aqueous phase (Sample)	37 mL
Organic phase (Toluene)	2.5 mL
MIP wash Wash solution	10 % deionised water in methanol
Extraction time	15, 30, 60 minutes

Trouble shooting with HPLC

How to start the instrument

Firstly the mobile phase should be sonicated for at least 40minutes. Switch on the detector, the HPLC instrument then the computer. Immediately when it starts pumping, the purge button must be opened in order to remove the bubbles. Wait until there is no more air bubble. Purging should be done for at least 5-10minutes. If there is an air bubble inside the instrument it can be noted by the fluctuation/instability in the detector. To know where the bubble is located in the instrument, first can switch off the pump, if the numbers in the detector still keep on changing then will know that the bubble is stuck in the detector but if not then it should be somewhere perhaps in the column.

To get read of that bubble, the column should be disconnected so that the pump could be increased to high flow rate. But if the bubble still is not coming out then ethanol should replace the current mobile phase and pumped through the system as the bubble does not dissolve the bubble therefore ethanol can remove the bubble from the system. However ethanol should not pumped for a long time but at least 30-40min and that should be done without column connected. After pumping ethanol to the system, replace the current mobile phase and pump it for 30-40min to remove ethanol from the system and that should be done before column connected again. When the column connected after that period and the detector still unstable, the column can be disconnected from the other end and pump for some time and again can be connected. Another thing, the bubbles can be overcame by filtering the mobile phase and then sonicate it. It really reduce the problem of the bubbles to the system.