INTRODUCTION

Molecularly imprinted polymers have found varied applications in different areas of science. MIPs have been used different areas of research as affinity separation materials, antibody binding mimics, enzyme mimics and as sensors (Müller, 1993; Kriz D, 1996; Lübke, 1998; Haupt, 1998, 1999; Arnold, 1999; Jenkins, 2001, 2005; Lavignac, 2004). In the present study the MIPs synthesized were used as:

1. Molecular sieves in solid phase extraction methods
2. Developing immunoassays

MIPs could not only help as detectors of environment toxicants but also as one step clean up sorbents /sieves for remediation of surface borne residues in fruits, vegetables, water and soil. Current sample clean-up methods like solid phase extraction methods are very fast and inexpensive, but exhibit lack of selectivity. Methods based on immunoaffinity or antibodies are very selective, expensive and are not suitable for harsh environments (Hennion, 2003; Pichon, 2002).

Solid-phase extraction (SPE) or sorbent extraction refers to physical extraction process involving a liquid and a solid phase. It consists of two immiscible phases. However, the extracted phase in this case is a solid material or organic liquid immobilized on the solid bed. SPE is the most widely used sample preparation method/technique in the past (Shahtaheri, 2005, 2007, 2008). Solid phase extraction is comparable to simple liquid chromatography where the sorbent is the stationary phase and mobile phase is aqueous while extraction and washing step and organic mobile phase is used for elution of analytes. During the extraction step, an aqueous sample is passed through the sorbent material. Target analytes are trapped on the sorbent. The trapped analytes are then eluted by passing a small volume of organic solvent. The elution solvent used should be able to extract the analytes (high performance) with low solvent volumes.
(3-5 ml), should be non-toxic and compatible with the instrument to be used for analysis. SPE has proven to be effective for cleaning, extracting and concentrating pollutants in analyses of environmental samples. The ideal sample preparation methodology is fast, accurate, cost effective and easily adaptable for field work. In scientific literature, several methods for extraction of pesticides from soil samples have been reported (Tsipi, 1999; Seng, 2003; Sharif, 2006).

Typical Solid phase extraction consists of four steps

- **Conditioning the sorbent.** The surface area of the sorbent is increased in this process opening up hydrophobic chains in a C18 sorbent with methanol.

- **Percolating the sample/extraction of the target analytes from the sample by addition of solvent.**

- **Rinsing to remove interfering compounds.** Here a small amount of organic solvent is passed but this should not interfere with the analytes.

- **Eluting the analyte with elution solvent**

There are several materials available as sorbent for solid phase extraction in cartridge form, column and discs configuration. 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 HPLC, that may be adsorbents such as silica gel or Florisil™, reversed-phase materials (e.g. with chemically-bonded octadecylsilyl ("ODS" or "C18" groups) or ion-exchange media (e.g. with bonded aminopropyl or phenylsulfonic acid moieties). The packing material is held in place within a polymer (usually polypropylene of a serological grade) column by porous frits, also constructed of a polymer material.
Molecularly imprinted polymers have proved to be an excellent replacement for the above traditional extraction materials (Lanza, 2001; Caro, 2006). MISPE is currently the most advanced application area with respect to the adoption of MIP-based technologies by the wider scientific community. Molecularly imprinted polymers have gained importance in recent years as SPE sorbents for the pre-concentration and extraction of analytes of interest in environmental samples.

In our study, the MIPs synthesized were intended to be used for extracting analytes from environmental samples. Their selectivity and recognition mechanisms were further evaluated using soil and milk samples. As the bulk polymer for heptachlor with MAA functional monomer and DDT sol-gel polymer for DDT showed good imprinting effect (equal to or above 2) for the target analyte as discussed in Chapter 3, were used in the SPE studies.

Another very important and lesser explored area is the application of MIPs to develop immunoassays for the environmental samples. First molecularly imprinted sorbent assay (MIA) was analogous to the competitive solid-phase radioimmunoassay (Vlatakis, 1993), MIP was used in place of polyclonal antibodies. There were many assays developed which employed chemiluminescence or fluorescence detection for better sensitivities (Piletsky, 1997; Haupt, 1998). MIPs, owing to their specificity, ease of preparation, low price, and high chemical and physical stability, could provide a useful complement or alternative to biological receptors for use as recognition elements in such assays. This is especially true in cases where a natural receptor does not exist or is difficult to obtain in large quantities. We therefore employed the molecular imprinted polymers prepared for DDT and heptachlor to develop ELISA.

5.1. MATERIALS

Pesticide standards were a gift from CSIRO, Australia. The compounds used for study were heptachlor, heptachlor epoxide, DDT, DDE, DDD and endosulfan. Methanol HPLC grade was obtained from RANKEM, INDIA. Milli Q water was used for chromatography. PHARMACIA
Chapter 5: Application of MIPs in Solid Phase Extraction and Immunoassays for OCPs

LKB pump (Sweden) was used to maintain the flow during extraction from SPE cartridges. MIPs synthesized using bulk polymerization for heptachlor and Sol-gel polymers for DDT were used for MISPE.

5.1.1 HPLC analysis

A HPLC Instrument - Shimadzu, (SCL-10A VP), KYOTO, JAPAN with Pump - LC-10AT, Detector- SPD-10A VP and System control- SCL-10A VP was used. Software- Shimadzu LC workstation CLASS-VP was used.

Chromatographic separation was performed on a PARTISIL 5 ODS-3 WCS Analytical column 4.6mm × 250mm (Whatman International Ltd Maidstone, England). Mobile phase of a mixture of methanol : water (75:25 v/v) was delivered at a flow rate of 1.0 ml min⁻¹ with detection at 220 nm. The mobile phase was filtered through a 0.45 μm membrane filter and degassed before use. The injection volume was 20 μl and analysis was performed at an ambient temperature of 25°C.

5.1.2 MISPE Method Development and Optimization

5.1.2.1 Sample preparation

Two samples namely garden soil and milk were selected to evaluate the MIPs synthesised; as SPE material and as synthetic antibodies.

Untreated garden soil was collected from the local horticulture department and sieved to obtain particles of < 3mm size. The spiked samples were prepared by adding a known volume of the pesticide stock solution to achieve a final concentration of 200 ng 10 g⁻¹ of soil. A known volume of acetone was added to the sample and kept in a horizontal shaker and mixed for 24 h. These spiked samples were left on the laboratory bench with intermittent swirling for three days before extracting the pesticide. The pesticide was extracted by sonicating the spiked soil samples.
in 20 ml of hexane: acetone (1:1 v/v) for 30 min. The sample was filtered and the elute was evaporated to dryness by flushing with nitrogen gas.

Milk samples for the study were procured from local market and were spiked with pesticide stock solution to reach a final concentration of 200 ng per 100 ml. The samples were extracted with 100 ml of dichloromethane. The samples were evaporated dry by flushing with nitrogen gas.

5.1.2.2. MISPE Column Preparation

To prepare the MISPE columns, 150 mg of MIPs were weighed into a test tube and were slurried with 3.0 ml acetonitrile. The slurry was transferred to the empty SPE cartridges and was left to settle under gravity to ensure the particles were uniformly packed into the cartridges (Figure 5.1). A frit was placed above the MIP sorbent bed to avoid disturbing the bed on solvent addition.

![Figure 5.1](image)

Figure 5.1: (a) Preparation of MISPE columns

(b) Steps in MISPE procedure (Source; Qiao, 2006)
5.1.2.3. MISPE procedure

The columns were attached to an SPE vacuum manifold, connected to a vacuum pump. The solid phase extraction was carried out according to the method described by Deans et al. (1993). The SPE cartridges were pre-equilibrated and solvated with two column volumes of ethanol followed by two column volumes of 1% aqueous methanol. Two millilitres of the sample extracts in chloroform were passed through the cartridge, dried with nitrogen gas. The interfering materials were removed by passing 5 ml of 1% aqueous methanol through the cartridge. The pesticide was extracted with 2ml × 3 times of methanol, evaporated to dryness with nitrogen gas and made up to 1 ml with chloroform and analyzed quantitatively using HPLC.

As mentioned in Chapter 3, section 3.1.3.1.1, polymers were extracted with excess methanol before rebinding till the presence of template in the polymer was zero, however, the MISPE cartridges were washed with successive methanol fractions (3×1 ml each) (Zander 1998; Baggiani, 2001) for both heptachlor and DDT. The eluted samples containing pesticides were injected and characterized using RP-HPLC.

5.1.2.4. Validation experiments

The MISPE developed were validated by application to soil and milk samples. As per the ICH (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines, the method validation parameters checked were linearity, accuracy, and precision, limit of detection and limit of quantitation. Pesticide recovery studies were performed for spiked soil samples (three replicates) at a spiking concentration of 200 ng 10g$^{-1}$ of sample. The intermediate precision (variations on repeating the same method) was calculated as the relative standard deviation (RSD) of the samples. The linearity of the calibration curves was evaluated at a concentration range between 50 -200 ng 10g$^{-1}$ spiked soil samples using the standards prepared in chloroform. Calculations of pesticide recoveries were
done by using the calibration curve. The calibration curve was developed by plotting peak area versus pesticide concentration. Linearity was expressed as the squared correlation coefficient ($R^2$). The limit of detection (LOD) and the limit of quantification (LOQ) were determined for the compounds.

**Limit of Detection and Limit of Quantification**

The Limit of Detection (LOD) is the smallest concentration of the analyte that gives the measurable response. LOD was calculated using the following formula

$$\text{LOD} = \frac{(3.3 \times \text{standard deviation})}{\text{Slope of calibration curve}}$$

The Limit of Quantification (LOQ) is the smallest concentration of the analyte, which gives response that can be accurately quantified. LOQ was calculated using the following formula

$$\text{LOQ} = \frac{(10 \times \text{standard deviation})}{\text{Slope of calibration curve}}$$

**Accuracy and Precision**

The precision of the method was demonstrated by interday and intraday variation studies. In the intraday studies, 3 repeated injections of standard and sample solutions were made in a day and the response factor of compounds peaks and % RSD were calculated. In the interday variation studies, 3 repeated injections of standard and sample solutions were made on 3 consecutive days and response factor of compounds peaks and % RSD were calculated.

**5.1.3. MIP Immunoassay**

MIP powders (20 mg) were suspended in 1 ml methanol : water (1:1, v/v) containing 0.2% PVA. 50 µL of particle suspension was applied to each well of polystyrene micro titer plates (96 wells) and dried at 70°C for 30 min. Other steps were similar to the colorimetric ELISA using egg yolk antibodies described in section 2.2.1.3 Chapter 2. The washing steps were performed gently, and
the plates kept reversed on filter paper to get rid of the residual washing buffer. The optical density of the colored product was measured using an ELISA plate reader.

**5.2. RESULTS AND DISCUSSION**

**5.2.1. Molecularly Imprinted Solid Phase Extraction (MISPE)**

**5.2.1.1. MISPE for DDT**

MISPE for DDT was performed using cartridges filled with sol-gel polymer. For spiked soil samples, elution pattern from different MISPE steps is shown in Figure 5.2a. The amount of DDT in elutes was calculated from the calibration curve (Figure 5.2b). As can be observed from Figure 5.2a, up to 80% of the total extracted DDT was eluted in the second step and only 2% of the analyte was eluted in the 3rd or the last elution step. In the first elution fraction, the analyte was almost nil.

![Figure 5.2a. Percent recovery of DDT from MISPE steps from spiked soil samples:](image)

W- washing, E1- Elute 1, E2- Elute 2, E3- Elute
This can be explained based on the solubility of the OCPs in different solvents; after washing step with 5% aqueous methanol to remove the interfering materials, the environment in the column is primarily hydrophilic. When methanol is passed through the column it replaces the water from the polymer cavities. In the second step, the analyte gets eluted with 100% methanol.

The chromatogram of elute from MIP cartridge showed the retention time of 22 minute. The chromatogram for elute from spiked soil and milk samples is given in Figure 5.3. Percentage recovery of DDT from spiked soil samples and milk samples were was 88 ± 3.5%, and 84 ± 4.3%. For preparing sol-gel MIP for DDT; EBP was used as a template analogue, for MISPE using EBP as template analogue is advantageous. In case of template bleeding (elution of residual template during MISPE) the recovery of the analyte from column will not be affected. Furthermore, in molecules like DDT, availability of template analogues also helps in avoiding the handling of DDT for polymerization, which is toxic. For DDT sol-gel MIPs, the binding sites are created after the removal of EBP and the cavity binds DDT based on structure recognition and non-covalent interactions between DDT and the binding sites. The major weak forces are π-π interactions between DDT and BTEB molecule. Imprinted polymer for the isolation of the mycotoxin, Zearalenone was prepared using a structure mimic and was employed for the MISPE from the corn samples (Mausia, 2011).
Figure 5.3. HPLC separation of DDT (retention time-22 min) elutes from MISPE a) DDT standard b) DDT from spiked soil sample c) DDT from spiked milk sample (d) control sample
5.2.1.2. MISPE for Heptachlor

Figure 5.4 a,b show the % recovery and calibration curve of heptachlor from MISPE columns respectively. The template was rebound based on the non-covalent interactions. For heptachlor MISPE, the recoveries were about $80 \pm 3.2\%$ for spiked soil samples and $76 \pm 4.2\%$ for spiked milk samples.

Figure 5.4. (a) Percent recovery of heptachlor from MISPE steps from spiked soil samples: W- washing, E1- Elute 1, E2- Elute 2, E3- Elute

Figure 5.4b. Calibration curve for Heptachlor from RP-HPLC
The chromatogram for elute from spiked soil and milk samples is given in Figure 5.5.

Figure 5.5. HPLC separation of Heptachlor (retention time-20 min) elutes from MISPE
a) Heptachlor standard b) Heptachlor from spiked soil sample c) Heptachlor from spiked milk sample (d) control sample

5.2.1.2 Method Validation

MISPE method was validated for LOD, LOQ, intraday and interday precision. The data is presented in Table 5.1. The standard deviation values are below 10% and are acceptable.
Table 5.1. Calibration and detection limits of the selected pesticides

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% RSD Intra Day n=3</th>
<th>LOD mg l⁻¹</th>
<th>LOQ mg l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inter day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDT</td>
<td>10.4</td>
<td>6.0</td>
<td>18.4</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>3.18</td>
<td>0.7</td>
<td>2.4</td>
</tr>
</tbody>
</table>

5.2.2. Comparison with conventional solid phase extraction

MISPE method developed was compared with commercial solid phase extraction. As OCPs are hydrophobic in nature, C18 cartridges were employed to perform SPE. The comparison table (Table 5.2) shows the recovery for DDT and heptachlor from MISPE and SPE cartridges.

Table 5.2. Comparison MISPE with commercial C18 solid phase extraction columns

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% Recovery ± % RSD n=3 (SPE) C18</th>
<th>% Recovery ± % RSD n=3 (MISPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil</td>
<td>Milk</td>
</tr>
<tr>
<td>DDT</td>
<td>92.9 ± 3.26</td>
<td>95.6±2.9</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>96.2 ± 4.47</td>
<td>92.1±3.4</td>
</tr>
</tbody>
</table>

The recovery values were high for C18 cartridges (above 90 %) compared to the MISPE columns which depends on the strength of binding interactions of the sorbent matrix and on imprinting effect in case of MIPs.

For efficient MISPE, polymerization of MIP is the initial step of successful selectivity. In our study, imprinting was more for DDT sol-gel polymer was most efficient followed by
Heptachlor-MAA bulk polymer. Novel technologies can be developed to synthesize or use template analogues which may provide a higher imprinting, leading to a more selective and sensitive MISPE.

MISPE methods have been reported for many pesticides from different food samples generally fruits and vegetables. MIP polymers prepared for traizines were applied for simultaneous extraction of several related analytes as atrazine, propazine, simazine and terbutylazine and related metabolites (Bjarnason, 1999; Chaupis, 2004). These reports show analyte recoveries lesser than 80% for MISPE.

A MISPE technique has the ability of selectively isolating specific compounds or their structural analogues from a complex matrix. The application of these synthetic polymers as sorbents allows not only pre-concentration and cleaning of the sample but also selective extraction of the target analyte, which is important, particularly when the sample is complex and impurities can interfere with quantification.

5.2.3. MIP Immunoassay

5.2.3.1. DDT Immunoassay

The MIPs synthesised were used as synthetic antibodies to format a competitive immunoassay. Colorimetric competitive ELISA was performed using bulk polymer for heptachlor and sol-gel polymer for DDT. As reported previously (Piletsky, 2000) microplates coated with MIP polymers can be used successfully in an enzyme-linked assay using competition between free pesticide and HRP-pesticide conjugate.

The results, presented in Figure 5.6a, clearly indicate high specificity of the imprinted polymer for DDT. The IC$_{50}$ values calculated for DDT was 250 ppb. The binding of the analyte depends on the weak forces as hydrophobic interactions, van der Waals interactions and steric selectivity. As studied previously, the binding capacities for sol-gel polymers were best among all the polymers synthesized; with imprinting factor of 2.8. Figure 5.6a shows the standard graph
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of competitive assay. When compounds structurally related to DDT were tested in the competitive assay, their cross-reactivities were in the range of 50% for DDE and 46% for DDD.

![Figure 5.6a. Standard graph for colorimetric ELISA with DDT sol-gel polymer](image)

**5.2.3.2. Heptachlor Immunoassay**

The standard graph for heptachlor immunoassay is shown in Fig 5.6b. The IC$_{50}$ was 3 ppm. The cross-reactivities were calculated to be 45% for heptachlor epoxide and 38% for endosulfan.

![Figure 5.6b. Standard graph for Colorimetric ELISA with Heptachlor-MAA-EGDMA polymer](image)
5.1.4. Application of the immunoassay to real samples

In an attempt to evaluate the suitability of the assay for measurement of real samples, DDT and heptachlor concentrations in spiked milk samples were determined. Pesticide was added at different concentrations to milk. The samples were then measured in triplicate using 96-well plates. The analyte concentration in the spiked samples was determined from the standard graph (Figure 5.6). The concentrations determined/measured from the standard graph were plotted against the known concentration of the analyte in the spiked samples. The correlation between known and measured concentrations was satisfactory with the $R^2$ values of 0.924 for DDT and 0.856 for heptachlor in the linear range, indicating the efficiency of the method developed.

MIP immunoassays have been developed for many drugs and few environmental contaminants (Irene, 2000, Haupt, 1998). The detection methods used were radioimmunoassay and chemiluminescence detections respectively. For estradiol MIP by Irene et al. the IC$_{50}$ values obtained was 60 ppb. The cross-reactivities reported for similar compounds were below 50% compared to the natural antibodies for estradiol. This shows the higher selectivity of MIPs compared to the natural polyclonal antibodies. Fluorescence detection based immunoassay have been developed for herbicide 2,4 dichlorophenoxy acetic acid (Haupt, 1998) with IC$_{50}$ values of 0.2 ppm. The performance of assay depends on the binding capacity of MIP. The DDT MIP assay was more sensitive compared to heptachlor MIP due to the difference in their binding capacities which can be correlated to their imprinting factors.

5.4.3. Comparison between Natural (polyclonal) and Synthetic antibodies (MIPs)

We compared the immunoassays based on IgY antibodies and ELISA developed with MIPs (synthetic antibodies) synthesized for OCPs. The MIP immunoassays were about 100 times less sensitive compared to the ELISA with IgY antibodies with the IC$_{50}$ value upto 10 ppb (Table 5.3).
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Table 5.3. Comparison of IC_{50} values for ELISA based on IgY and MIP

<table>
<thead>
<tr>
<th>Method</th>
<th>( R^2 ) value</th>
<th>IC_{50} value (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DDT</td>
</tr>
<tr>
<td>IgY</td>
<td>0.861</td>
<td>0.881</td>
</tr>
<tr>
<td>MIP</td>
<td>0.924</td>
<td>0.856</td>
</tr>
</tbody>
</table>

The selectivity of the MIPs have been shown to be lesser compared to the polyclonal antibody based assays. This variation in the sensitivity may be due to the following reasons.
1. Polyclonal antibodies may have many natural epitopes for analyte recognition, but MIPs have only one binding site depending on the presence of functional groups on template molecules. Thus, MIPs can be used as screening material for high throughput samples.
2. The analytes used in the present study are highly hydrophobic and the use of water in the immunoassays limits the sensitivity of the method. However, the ease of preparation, cost and time of preparation makes MIPs a preferred system for the detection of OCPs.

5.4. CONCLUSIONS

As shown in this study, MIPs can be successfully used as selective sorbents to clean up and pre-concentrate contaminants of natural or from food samples.

In the present work, we applied the synthesized polymers as sieve materials to extract the pesticides from environmental samples. MISPE method was developed for heptachlor and DDT. The recoveries obtained were above 80% for both the pesticides. From the examples reported, it is evident that MISPE is a potentially competitive technique to traditional solid phase extraction techniques due to its selectivity, and has the following advantages over the conventional SPEs:
1. High affinity and specificity
2. Customised and low cost of preparation
3. Re- usability.
Many polymerization methods and extraction formats make MISPE a very versatile technique, suitable for analytes of different origin and nature. A careful choice of the template structure offers the possibility to develop MISPE methods suitable for the simultaneous extraction of many related analytes, opening the way to sample screening for whole gamut of analytes. The main problem affecting MISPE, i.e. the undesirable template leakage during the analyte elution step, can be avoided using the mimic template method. On these premises, extraction of food contaminants is destined to become a relevant application of MISPE as well as the extraction of analytes from clinical, environmental and pharmaceutical matrices.

Further, competitive assay for the OCPs heptachlor and DDT was developed which is based on MIPs (synthetic Antibodies) in place of polyclonal antibodies. The assay was performed as standard competitive ELISA method. The IC$_{50}$ values of upto 1 ppm were achieved for both the pesticides. Although this approach is unlikely to be universally applicable, it could provide a useful assay system in many instances. These findings increase the potential of molecularly imprinted polymers for immunoassay applications.