4. Analysis of Endocrine Disruptor and Suspected Endocrine Disruptor Pesticides by MEPS-HPLC-UV and its Application

4.1 Introduction

Endocrine disruptors (ED) are the substances that mimic the human hormones, and block their receptive sites in our bodies, leading to the disruption of normal activity and functioning of the endocrine system [1]. A variety of chemical compounds including hormones, plant derivatives, pesticides and chemicals used in the plastics industry and other industrial by-products which are rapidly finding way into our food chain, can act as endocrine disrupting compounds. ED substances pose a serious threat to our intelligence, our ability to procreate and general well being [2]. As a result it becomes imperative that ED substances are identified and sensitive methods are developed for their detection in food and environmental matrices. In the present study, it has been aimed to analyze ED pesticides propazine, terbutryn and aldicarb alongwith a suspected endocrine disruptor; dimethoate.

Several methods are available in the literature for the determination of these pesticides in food stuffs [3-9]. Many studies are available in the literature which analyze one or more of these pesticides using different techniques like HPLC with UV detection [7, 8, 16-26] or fluorescence detection [3, 27, 28] or diode array detection [11]; some papers deal with LC coupled with MS (mass spectrometry) [5,6, 13, 25, 28- 34] ; a few methods based on use of micellar electrokinetic chromatography (MEKC) [35,36] are also available. Gas Chromatography (GC) methods are also available [12, 14, 37]; GC coupled with MS [5, 27], ECD (electron capture detector) [36]. Molecule imprinted polymer technique [9,38] extraction spectrophotometry [39], kinetic spectrophotometry [10], fiber optic
immunosensor [40], biosensor [43], chemiluminescence detection [42] and time resolved fluoroimmunoassay [43].

Figure 4.1: Structure of Endocrine disruptor pesticides

Extraction is an important step for the pesticide residue determination in various matrices. Several methods are reported in the literature for this purpose namely, SPE [17, 21, 23, 24, 29, 31, 37], Microwave assisted extraction i.e. MAE [16, 18, 20], dispersive liquid liquid microextraction [7], liquid solid extraction [15] and solid phase microextraction (SPME) [9].

Of the many techniques available, solid phase extraction has been the favoured method for the pre-concentration of many of these pesticides. But the need of the hour is to develop swifter and more selective sample preparation and clean-up techniques requiring
lesser sample volumes. A new pre-concentration technique; MEPS (microextraction by a packed sorbent) [44] is a leap forward in this direction. MEPS is a novel, miniaturised version of SPE. MEPS kit differs from commercial SPE cartridge in that the only 4 mg solid sorbent is inserted into a syringe on which adsorption takes place as a plug [45], this miniaturisation greatly reduces the sample volume used for one analysis from the millilitre to the microlitre range, hence, the eluent volumes are of the magnitude to be injected into LC or GC systems [46]. MEPS can be fully automated and is suitable for the rapid analysis of biological samples. MEPS technique has been used by Prieto et al in a new procedure for the multiresidue analysis of 41 organic pollutants in water samples through large volume injection-GC-MS (LVI-GC-MS) [47]. MEPS has also been used successfully for the drug analysis in various matrices [48-52]. MEPS technique has been used by Rani et al for liquid chromatographic drug analysis in plasma and urine samples [53] and LC and GC-MS determination in plasma and urine samples [54]. The aim of the present study is to develop a new and improved method for the analysis of four ED pesticides by using MEPS as a sample preparation technique with LC-UV for the first time. This method is a novel one which determines the pesticides; dimethoate, aldicarb, propazine and terbutryn by LC-MEPS technique in a single run. The major advantages of using the MEPS technique are enlisted below:

1. MEPS results are comparable to SPE ones but is faster and more convenient than SPE.

2. It uses small sample size, which is advantageous for the analysis of biological fluids.

3. The small elution volume ensures that the whole sample can be analysed through HPLC rather than a part of it as in conventional extraction methods.
4.2 Experimental

4.2.1 Standards and Reagents

Pesticide standards of aldicarb, dimethoate, propazine and terbutryn were obtained from Reidel de Haën (Germany). LC grade acetonitrile was purchased from J.T.Baker, USA. Triply distilled water was used for all purposes. Water was demonized (Riviera, SCHOTT DURAN, Mainz, Germany) before use. All the solvents were filtered through 0.45 µm, Nylon-6,6 membrane filters (Rankem, New Delhi, India). Separate stock solutions of dimethoate, aldicarb, propazine and terbutryn with concentration of 100 µg mL\(^{-1}\) were prepared by dissolving the pesticides in methanol and were stored in refrigerator at -4ºC. Further dilutions were done as per requirement.

4.2.2 Apparatus and chromatographic conditions

A Dionex P680 HPLC pump, Dionex Acclaim 120 C\(_{18}\) RP analytical column (4.6 x 250mm; 5µm) and Dionex UVD 170U detector were used for the chromatographic determination of the pesticides. The detector wavelength was set at 220 nm. Chromeleon computer program was used for the acquisition of data. The pesticides were separated under isocratic flow of ACN/water in the ratio 60:40 at a flow rate of 1 mL min\(^{-1}\). Microextraction was carried out on a BIN (Barrel Insert and Needle Assembly) containing 4 mg of solid-phase silica-C\(_{18}\) material, inserted into a 250 µL gas-tight syringe from SGE Analytical Science (Melbourne, VIC, Australia). The sorbent particles are 45µm in size with porosity 60 Å.

4.3 Procedure

4.3.1 Sample preparation
4.3.1.1 Urine samples

Urine samples were collected from healthy volunteers and stored in polypropylene tubes and stored at -4°C until the time of sample treatment. It was brought to room temperature and centrifuged for 5 min. Spiked urine samples were prepared by adding required concentration of analytes to 1 mL of centrifuged urine. Then, the samples were extracted and analyzed. Calibration curves were obtained for the concentration range 1-500 ng mL\(^{-1}\) and 5-500 ng mL\(^{-1}\) for aldicarb.

4.3.1.2 Soil samples

The soil samples were prepared by weighing 2 g of powdered and sieved soil. The known concentrations of analytes were added to the soil. The spiked soil was allowed to dry overnight at room temperature. 10 mL of HPLC grade methanol was added to this sample and sonication was done for 10 minutes. The sample was further filtered on Buchner funnel under suction and 2-3 washings were given with methanol. The filtrate was then filtered through membrane filters. The methanol was allowed to evaporate at room temperature. The residue was dissolved in 1 mL of distilled water. The spiked samples were prepared by adding known amount of analytes to the residue in the concentration range 1-500 ng mL\(^{-1}\) and 5-500 ng mL\(^{-1}\) for aldicarb.

4.3.1.3 Tap water samples

The tap water sample was taken from our lab in Pyrex borosilicate amber glass containers, which were rinsed with triply distilled water. The samples were then filtered through Nylon-6,6 membrane filters in a filtration assembly, sonicated to degas and were stored at -4°C . The spiked samples were prepared by addition of analytes with different
concentrations in the range of 1-500 ng mL\(^{-1}\) and 5-500 ng mL\(^{-1}\) for aldicarb.

4.3.2 Microextraction by packed sorbent procedure

Before use the MEPS needle was conditioned with 100 µL methanol and then with 100 µL water. The samples of the spiked urine, soil and tap water samples were drawn up and then discarded manually with downward push, through the syringe for 30 times. The multiple sample drawing action through the sorbent increases the extraction recovery of analytes. The samples were drawn slowly and carefully so as to get good percolation of the sample on the solid sorbent bed. The approximate speed of sample drawing was 20 µLs\(^{-1}\)(±5 µLs\(^{-1}\)).The sorbent was given washing with 100 µL water to remove interferences. The adsorbed analytes were then eluted with 30 µL of methanol and injected into the HPLC sample injection loop. In between the successive extractions through the MEPS needle, the C\(_{18}\) adsorbent in the BIN was washed with methanol (3 x 50 µL) and water (3 x 50µL) to remove the memory effects and to condition it for the next extraction. The same packing bed can be used for about 100 extractions before being discarded.

4.3.3 Method Validation

Calibration curves for spiked urine, soil and tap water samples were prepared in the range 1-500 ng mL\(^{-1}\) and 5-500 ng mL\(^{-1}\) for aldicarb. Good linearity (R\(^2\)>0.9918) [Table 4.1] was obtained for the selected concentration range. The calibration curves were described by linear regression equation:

\[y = mx + c\]

where y is the peak area, x is the concentration, m stands for slope and c is the intercept.
4.3.3.1 Extraction yield and precision

Extraction yield and precision assays were made at four different concentration levels of analytes, i.e. 1, 10, 100 and 250 ng mL\(^{-1}\) and 5, 10, 100 and 250 ng mL\(^{-1}\). The results are reported in Table 4.2. The results are satisfactory with extraction yield values being in the range 81.4-97.8%. The intraday precision was also satisfactory, with RSD values being ≤ 6.3% for all analytes. The experiments were conducted six times during the same day to obtain repeatability (intraday precision) and interday precision was obtained by repeating the experiments six times over six different days, both values are expressed as RSD% i.e. percentage relative standard deviation values.

4.4 Results and Discussion

4.4.1 Optimization of HPLC conditions

Endocrine Disruptor pesticides; aldicarb, dimethoate, propazine and terbutryn were separated on the HPLC system at these retention times: 3.54, 4.41, 8.5 and 12.3 min respectively by direct injection of samples. Various parameters like selection of suitable wavelength, mobile phase composition and flow rate were optimized. The mobile phase composition selected was ACN/Water in the ratio 60: 40 at a flow rate of 1 mLmin\(^{-1}\) at 220 nm. Calibration curves are linear in their respective ranges of pesticides. The correlation coefficient varied between 0.9918 and 0.9987. The absolute detection limits for these pesticides were in the range 0.05 and 0.60 ng mL\(^{-1}\) [Table 4.1] which were calculated as 3 x S/m, where S is the standard deviation and m is the slope of the calibration curve. The LOQ (limit of quantification) values were in the range 0.198 ng mL\(^{-1}\) and 0.79 ng mL\(^{-1}\). The extraction recoveries of the pesticides were calculated from
the comparison of peak areas of extracted samples to those of the analyte standard solutions. The representative chromatograms of blank and spiked samples extracted by MEPS are presented in Figs. 4.2, 4.3 and 4.4. The extraction recoveries of all the drugs were in the range 81.4-97.8% in the urine, soil and tap water samples [Table 4.2].

4.4.2 Optimization of MEPS conditions

The factors that affect absolute recoveries from the spiked samples vis-a-vis those from the calibration curves of standard samples were studied. These factors include time and velocity of the sample loading and volume of the eluting solvents. In MEPS, the recovery of the analyte greatly depends on the number of times a sample is drawn through the needle up and down. The volume of the eluting solvent was optimized at 30 µL. The elution efficiency of the solvents; acetonitrile, methanol, and different aqueous mixtures of methanol was tested. Methanol showed better results than acetonitrile and other solvent mixtures [Figure 4.5]. Methanol (30 µL) was found to give best elution efficiency. The effect of different washing solvents was studied on the percentage recovery of analytes [Figure 4.6]. Methanol and aqueous methanol mixtures were employed as the washing solvents to remove matrix interferences, but these resulted in increased leakage and lower extraction efficiency. Water (50 µL), drawn once, gave clean extracts, hence was used as washing solvent. Sample recovery was increased as the number of extraction cycles were increased up to 30, after which no significant increase in the recovery was observed [Figure 4.7].

The influence of pH on the extraction yield of the studied pesticides was investigated. The initial pH of the 10 ng mL⁻¹ solution was 5.7. For MEPS experiment, the water spiked with the pesticides was analysed on the C₁₈-BIN by varying the pH values: pH 2,
pH 4 (both adjusted with 0.1M Hydrochloric acid), pH 6 and pH 8 (both adjusted with 0.1 mol L^{-1} sodium hydroxide solution) were studied [Figure 4.8]. The extraction efficiency was found to be best at pH 5.7 as at lower or higher pH the pesticides were found to be unstable. Thus, the pH conditions for the experiment were optimized.

In the developed method, the impurities did not interfere in the regions of interest as no interfering peaks were seen in the blank chromatograms of urine, soil and tap water samples; hence the developed MEPS-HPLC-UV method is quite selective for the quantification of these pesticides. The effect of pH was also studied The LODs obtained for the pesticides from this method were also compared with other existing LC-UV methods and were found to be reasonably good [Table 4.3].

4.4.3 Selectivity

The method selectivity is a measure of its applicability for the determination of analytes in mixtures or various matrices without any interference from components showing similar behavior. During the application of present method to determine analytes in spiked samples using MEPS-HPLC-UV and comparison with blank samples, no significant interferences of endogenous substances were observed at the retention times of studied pesticides [Figures 4.1-4.3]. Therefore, the present method is highly selective.

4.4.4 Carryover and Matrix Effect

The carryover effect was studied on the column by making use of three successive aliquots of standard mixture having high concentration of all the analytes followed by successive aliquots of extracted blank urine, tap water and soil samples. A non-significant carryover effect (<0.1%) was observed. Assays were also carried out to ensure that the
small quantity of sorbent phase (4 mg) in the MEPS BIN can be effectively washed before the subsequent extraction to minimize the possibility of carryover. MEPS was washed three times with eluting solvent (3 x 50µL methanol) after each extraction of urine, tap water and soil sample spiked with a high concentration of analytes and then injected in the chromatographic system. The chromatograms of blank samples did not show significant matrix effect.

4.4.5 Comparison of method

The results of the present method are compared with the LODs obtained from earlier methods available in the literature (Table 4.3). The accuracy and precision of the present method are comparable to those from the reported liquid chromatographic methods used for the determination of these pesticides. The present method shows values of accuracy and precision which are comparable to the methods analyzing these endocrine disruptors. The present method reduces the extraction time as compared to the SPE [55]. This method gives better limit of detection than other LC-UV methods [8, 22, 23, 55-58]. The unique advantage of this procedure lies in the use of small sample volume which is < 1 mL while SPE and other conventional methods use 10-30 mL. The developed method is fast, simple and robust vis-a-vis the earlier methods.

4.5 Conclusions

The present method uses a new, sensitive and accurate sample preparation technique, MEPS for the analysis of endocrine disruptor and suspected endocrine disruptor pesticides in urine, soil and tap water samples. MEPS is more selective, time efficient and a robust technique as compared to the solid phase extraction. MEPS syringe containing
the packed sorbent can be used a number of times (100-200; according to the matrix used), whereas the SPE cartridge is meant for single use, this makes MEPS the more cost effective method. The present method has been successfully employed for the quantitative determination of the endocrine disruptor and suspected endocrine disruptor pesticides; dimethoate, aldicarb, propazine and terbutryn in various environmental samples like urine, soil and tap water samples.
Figure 4.2 MEPS/HPLC Chromatogram obtained from (a) blank soil and (b) spiked soil with; (A) dimethoate, (B) aldicarb, (C) propazine and (D) terbutryn at a concentration of 0.1 µg mL$^{-1}$
Figure 4.3 MEPS/HPLC Chromatogram obtained from spiked tap water with; A dimethoate, B aldicarb, C propazine and D terbutryn at a concentration of 0.1 µg mL$^{-1}$
Figure 4.4 MEPS/HPLC Chromatogram obtained from (a) blank urine and (b) spiked urine with; (A) dimethoate, (B) aldicarb, (C) propazine and (D) terbutryn at a concentration of 0.1 µg mL⁻¹.
Figure 4.5 Comparison of the elution efficiency of different elution solvents for the analytes on MEPS (C$_{18}$) syringe

Figure 4.6 Comparison of the analyte response to the use of the different washing solvents used on MEPS (C$_{18}$) syringe
Figure 4.7 Effect of number of extraction cycles on extraction efficiency

Figure 4.8: Effect of pH on extraction efficiency
Table 4.1: Linearity parameters of Endocrine Disruptor Pesticides from spiked urine, tap water and soil samples

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linearity Range (ng mL(^{-1}))</th>
<th>m (LOD, ng mL(^{-1}))</th>
<th>Urine</th>
<th>Tap water</th>
<th>Soil</th>
</tr>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Aldicarb</td>
<td>5-500</td>
<td>0.019(0.600)</td>
<td>0.018(0.580)</td>
<td>0.016(0.600)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.9954</td>
<td>0.9939</td>
<td>0.9918</td>
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<tr>
<td>Dimethoate</td>
<td>5-500</td>
<td>0.015(0.090)</td>
<td>0.018(0.085)</td>
<td>0.015(0.087)</td>
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<tr>
<td></td>
<td></td>
<td>0.9980</td>
<td>0.9974</td>
<td>0.9983</td>
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<tr>
<td>Propazine</td>
<td>1-500</td>
<td>0.041(0.068)</td>
<td>0.041(0.068)</td>
<td>0.039(0.070)</td>
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<td></td>
<td></td>
<td>0.9988</td>
<td>0.9988</td>
<td>0.9989</td>
<td></td>
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<tr>
<td>Terbutryn</td>
<td>1-500</td>
<td>0.041(0.056)</td>
<td>0.043(0.053)</td>
<td>0.040(0.058)</td>
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<td></td>
<td></td>
<td>0.9967</td>
<td>0.9972</td>
<td>0.9947</td>
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</tbody>
</table>

m: slope of the regression equation
Table 4.2: Results for the determination of extraction yield of endocrine disruptor pesticides from spiked urine, soil and tap water samples on HPLC-UV

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Amount added (ngmL⁻¹)</th>
<th>Extraction Yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Repeatability (RSD%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Interday precision (RSD %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>Soil</td>
<td>Tap Water</td>
</tr>
<tr>
<td>Aldicarb</td>
<td>5</td>
<td>89.3</td>
<td>83.6</td>
<td>85.4</td>
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<tr>
<td></td>
<td>10</td>
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<td></td>
<td>100</td>
<td>94.4</td>
<td>84.5</td>
<td>89.0</td>
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<td></td>
<td>250</td>
<td>95.7</td>
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<td>Dimethoate</td>
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<td>85</td>
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<tr>
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<td>Propazine</td>
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<td>97.8</td>
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<tr>
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<td>82.1</td>
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<td>93.9</td>
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<td>250</td>
<td>96.7</td>
<td>94.7</td>
<td>95.8</td>
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<sup>a</sup> Each value is a mean of 6 independent assays.
Table 4.3: Comparison of LODs of aldicarb, dimethoate, propazine and terbutryn with other LC-UV methods in literature and the present method

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Method Used</th>
<th>LOD in ng mL$^{-1}$</th>
<th>Reference</th>
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<td>Aldicarb</td>
<td>SPE-LC-UV</td>
<td>4</td>
<td>55</td>
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<tr>
<td></td>
<td>LC-LC</td>
<td>0.3</td>
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<tr>
<td></td>
<td>MEPS-LC-UV</td>
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<td>Present method</td>
</tr>
<tr>
<td></td>
<td>LC-UV</td>
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<tr>
<td>Dimethoate</td>
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<tr>
<td></td>
<td>MEPS-LC-UV</td>
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<td>Present method</td>
</tr>
<tr>
<td></td>
<td>LC-UV</td>
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<td>Propazine</td>
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<td></td>
<td>HPLC</td>
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<td>MEPS-LC-UV</td>
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<td>Present method</td>
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<td>LC-UV</td>
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<td>23</td>
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<tr>
<td>Terbutryn</td>
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<td></td>
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<tr>
<td></td>
<td>LC-LC</td>
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<td></td>
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References


