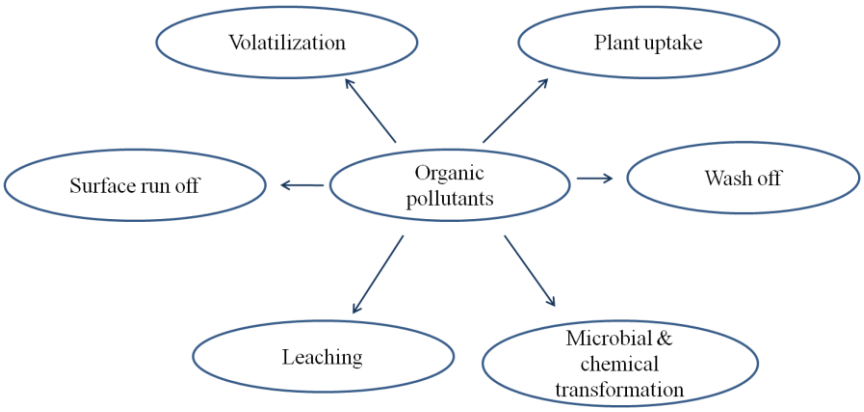
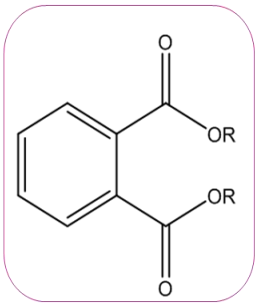
Chapter 3

Phthalate esters (PAE)



This chapter focuses on the comparison of some micro-extraction techniques for the determination of organic pollutants in water. Organic pollutants are a broad class of compounds that pollute soil, water, and air.



The class of organic compounds we studied was phthalate esters (PAE), which are potential endocrine disrupting agents. These are used as plasticizers in polymer industry. They are a concern for environment as they are a major class of contaminants polluting water, soil and sediments. The work focuses on comparing the micro extraction techniques and determining them in trace amount.

Chapter 3 Phthalate esters

3.1 Introduction

"Microextraction" evolved from the most conventional technique "extraction", a sample pre-treatment step developed as a solution for reduction in the consumption of extraction solvent. It has advanced over the years and has resulted in tremendous modifications to the basic extraction technique achieving shorter extraction duration, low organic solvent consumption, achieving lower detection limits to as low as ppb and 10^{-13} levels. These modifications are a result of the limitations and setbacks the different extraction techniques have suffered. It plays a crucial role prior to sample analysis for removing the undesired components in matrix and / or to enrich and isolate the analyte(s) of interest. It is used as a clean-up procedure for removing interferences such as impurities coming from various matrices such as water, plants, soil, reaction mixtures and sediment, etc. This comes as an advantage when most instruments cannot directly handle samples of these complex matrices. Therefore, the pre-concentration step becomes an important clean up procedure prior any analysis. The basis for all these microextraction techniques happens to be "Extraction" ***(figure 1).***

The liquid based extractions are mainly based on the principles of liquid liquid extractions (LLE). In these techniques, a water immiscible organic solvent is used as extraction solvent and the aqueous phase containing the target analytes (either spiked or real matrix samples) interact with each other. The sorbent based or solid based extractions are the ones in which an adsorbent is used to partition the target analytes (similar to that of column chromatography). In the case of SPE, an appropriate adsorbent is used based on the type of pollutants or analytes targeted, which is activated, loaded with the aqueous phase containing the analytes and then eluted with an organic extraction solvent and subjected to analysis directly. SBSE is based on the similar concept except that instead of a column, the sorbent is coated on a stir bar which can be directly introduced in the aqueous sample. Once the extraction is achieved, the stir bar can be dried and stirred in an extraction solvent, thus eluting out the target analytes in the extraction solvent.

In case of SPME, a microfiber made up of fused silica optical fibre coated with a hydrophobic polymer is used. This acts as the sorbent for the analytes to adsorb on its surface, which after they reach the extraction equilibrium are desorbed through the interface attached to a LC or GC system, thus making the technique solvent less.

In short, all these techniques use little to no extraction solvent, making the techniques eco-friendly. Higher extraction efficiency and lower detection limits are achieved.

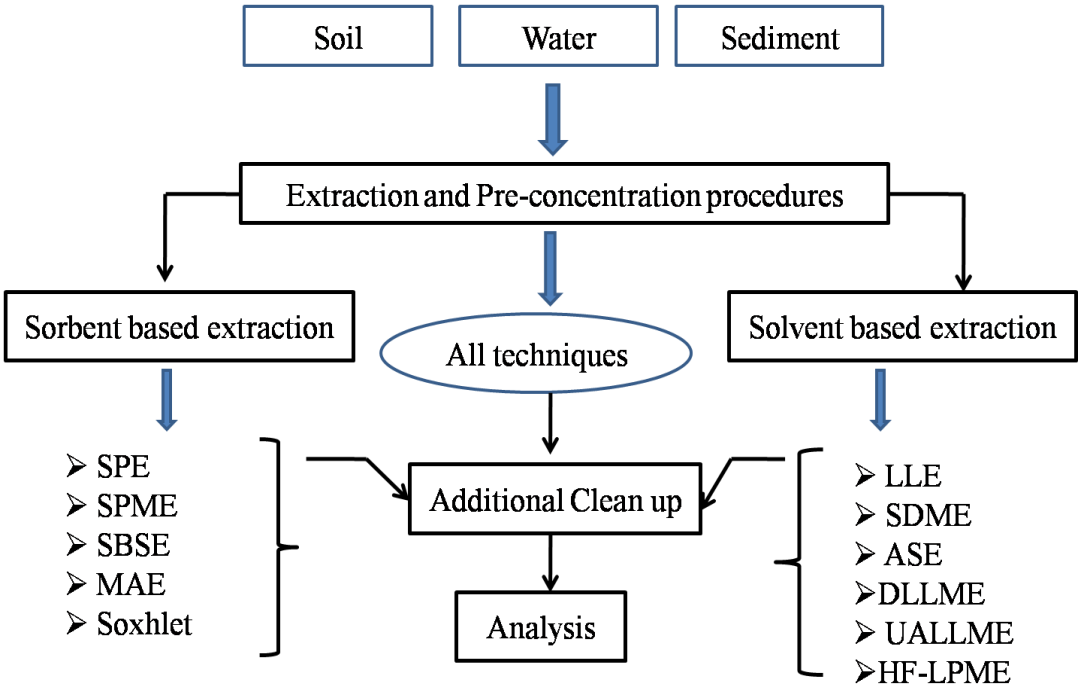


Figure 1 Microextraction technique

Phthalate esters, a class of organic compounds that have wide varieties of industrial, domestic and agricultural applications, styrene and rubber industry but so far the predominating applications are their use industrially as plasticizers to make plastics more flexible in a variety of polymeric materials (household and consumer products). Several phthalates have been identified and classified as endocrine disruptors possibly associated with known estrogenic and anti-androgenic activity. Because phthalate esters are not chemically bound to the plastics, they can be released easily from products and migrate into the food or water that comes into direct contact. Due to the widespread use of phthalates, they are considered as ubiquitous environmental pollutants ***(figure 2 and 3)*** [1]. Some phthalates have been included in the list of priority pollutants in several countries. For instance, the US Environmental Protection Agency (EPA) has established a maximum admissible concentration (MAC) value in water of 6 μgL^{-1} for di-2-ethylhexyl phthalate (DEHP). It is the most widely used phthalate in the world and it represents a quarter of the total production of plasticizers [2].

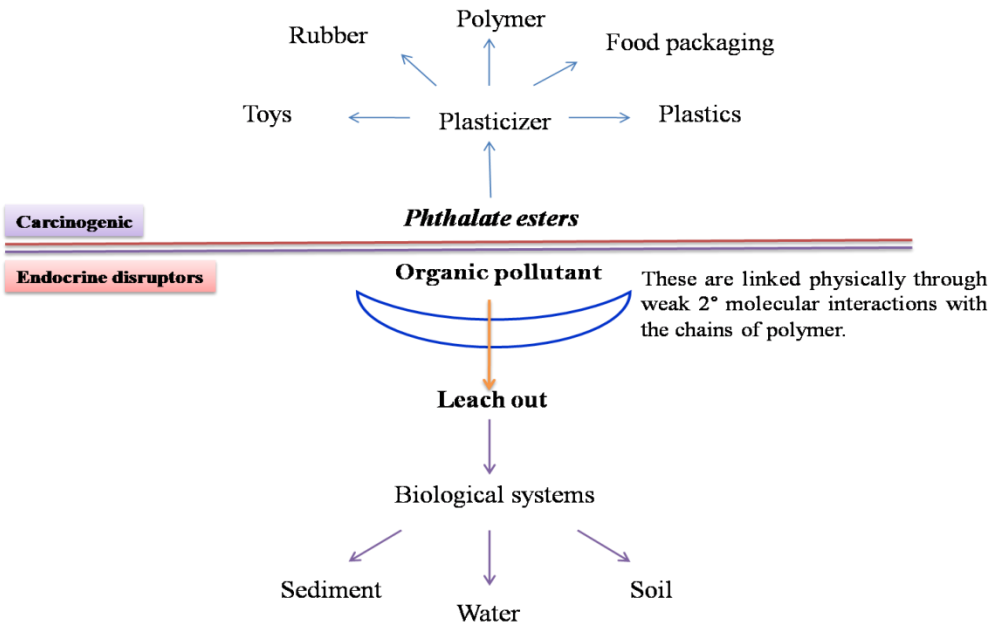


Figure 2 Phthalate ester in biological system

In order to determine trace levels of phthalate esters in water samples or any other matrix an extraction and pre-concentration step is often required prior to their analysis by an analytical technique. We used the methods after optimizing the parameters and compared them for real water samples for determination of phthalates.

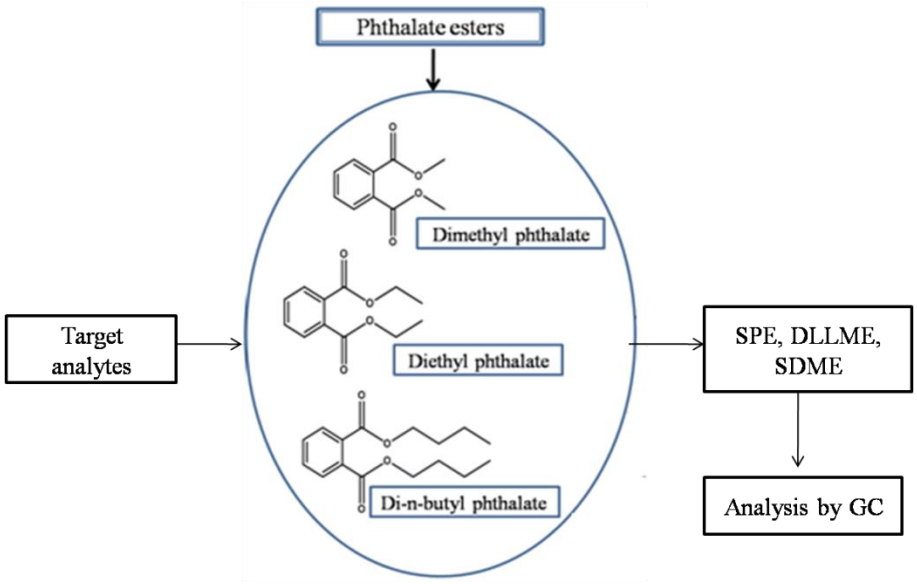


Figure 3 Target analytes - Phthalate ester

Phthalate esters have been determined in wine by solid phase extraction [3], in vegetables by means of stir bar sorptive extraction (coupled with GC-MS) due to their migration from plastics and packaging material, ultimately contaminating the vegetables and food material in contact. These contaminants can mimic the chemical and physical behaviour of natural hormones thus affecting the biological functions

that they carry out in animals and humans and hence belong to the class of endocrine disrupting agents [4]

Therefore, their determination at trace levels is necessary as plastics and packaging material have been used extensively in daily lives, ultimately becoming a source for phthalate ester leaching out in the environment.

The three techniques used are: Solid Phase extraction (SPE) is a sorbent based extraction technique ***(figure 4)*** wherein a solid adsorbent based on the type of analytes targeted is chosen.

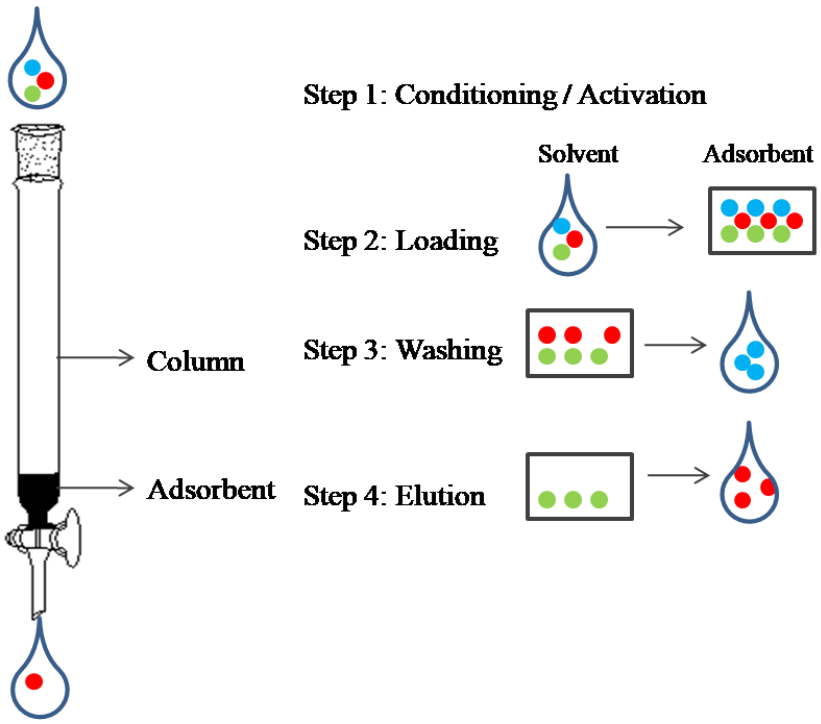


Figure 4 Schematic presentation for SPE

Dispersive liquid liquid microextraction (DLLME) is based on liquid liquid extraction forming a ternary solvent system wherein a mixture of disperser solvent and extraction solvent are rapidly introduced in an aliquot of water. The technique was introduced by Rezaee et al in 2006 ***(figure 5)*** [10].

The technique is based on liquid liquid extraction – ternary solvent mixture. This ternary mixture comprises of an aqueous phase, an organic immiscible extraction solvent and an organic dispersing solvent that has solubility in both the aqueous phase and organic phase. A mixture of extraction solvent and dispersing solvent is introduced in the aqueous phase containing the target analytes by a syringe rapidly in one stroke such that it results in the formation of a cloudy solution (an emulsion).

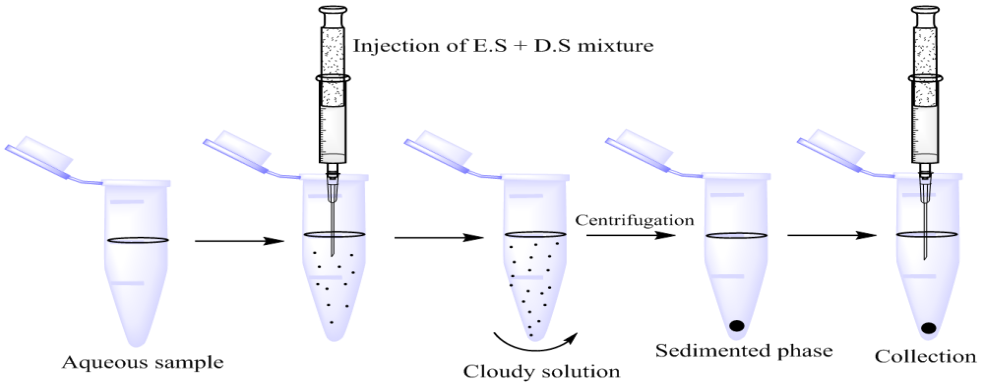


Figure 5 Schematic presentation of DLLME

Thus, resultant fine droplets of extraction solvent are formed in the solution which acts as an interface providing large surface area for the extraction of organic analytes from the aqueous phase into it. The advantage being that very low amount of extraction solvent required to extract target analytes, higher extraction recovery, reduced extraction time, reduced extraction solvent.

The third microextraction technique used was single drop microextraction (SDME) technique ***(figure 6)***, based on the conventional liquid liquid extraction. The amount of extraction solvent is just few micro litres (1 to 3 µL).

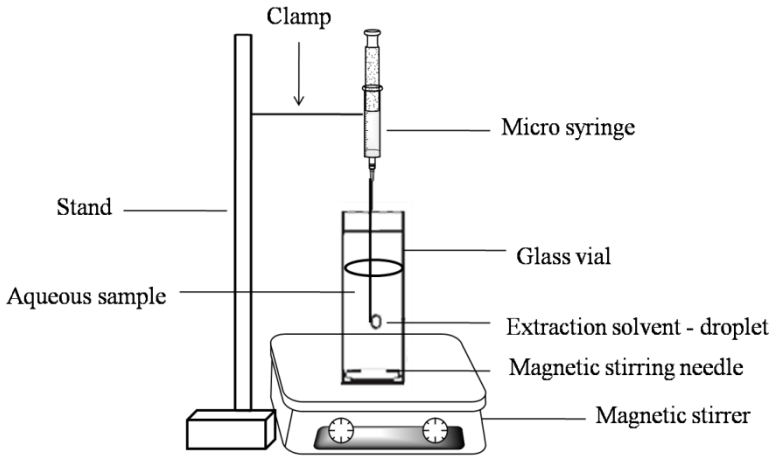


Figure 6 Schematic presentation for SDME

Parameters that were studied for the extraction technique:

- ✓ Effect of extraction solvent
- ✓ Effect of volume aqueous sample
- ✓ Stirring rate
- ✓ Extraction solvent drop volume
- ✓ Effect of salt addition
- ✓ Temperature

Single drop microextraction (SDME) developed in 1996 has been modified based on the requirement and thus has undergone modifications such as direct immersion (DI)-SDME, headspace (HS)-SDME and continuous flow microextraction based on application [13]. Over the years, a lot of changes have been made to the basic conventional liquid liquid extraction (LLE), in order to achieve lower extraction time, reduce consumption of organic solvents, emphasis on making the technique green, introduce a degree of automation, etc which has resulted in these microextraction techniques. Such modifications have been done in these microextraction techniques as well to enhance the scope of application of the microextraction technique. SDME [13] developed as result of advances made based on the short comings faced. It was in 1996, Liu and Dasgupta reported a drop-in-drop system to extract sodium dodecyl sulphate where a 1.3 µL microdrop of a water-immiscible organic solvent was immersed into a large flowing aqueous drop to accomplish the extraction process. During the same period, Jeannot and Cantwell developed a procedure which they termed as solvent microextraction. In this procedure, a droplet 8 µL of 1-octanol as extraction solvent was held at the end of a Teflon rod and suspended in a stirred aqueous sample solution. After extraction was achieved for a prescribed time, the Teflon rod was withdrawn from the aqueous solution; and the organic phase was sampled with the help of microsyringe, which was then injected in a GC system for analysis [13].

The short coming or limitation in this case was the use of Teflon rod used as extraction medium which could not be introduced directly in the GC system - resultant extraction and injection had to be performed separately using two different apparatus. To overcome this situation, Jeannot and Cantwell developed a method wherein a GC micro syringe was used in place of Teflon rod to hold the extraction solvent as droplet and then withdrawn back in the syringe once the extraction equilibrium was achieved [13]. Thus, the micro syringe served both as solvent holder and as syringe to be directly injected in GC system with use of just a single apparatus (micro syringe).

Headspace (HS) –SDME is similarly based on the use of micro syringe as sample holder except the droplet is held above the aqueous sample (in head space) and not immersed in it and after extraction directly introduced in the GC system [13]. Thus, with the development in these techniques, their application has been made in determination of organophophorous pesticides in water and fruit juice by Xiao et al

[14]. Similarly, chlorophenols have been determined from water by SDME by Saraji et al [15]. With an aim to achieve higher extraction with the use of minimum solvent, we have used SPE, DLLME and direct immersion (DI)-single drop microextraction in our work.

3.2 Materials and instrumentation

All the chemicals and solvents used for the sample preparation and analysis were of analytical grade and used as purchased. All the phthalate esters, namely dimethyl phthalate (DMP) and diethyl phthalate (DEP) were of analytical grade, 99.9% purity purchased from Merck. Analytical grade Di-*n*-butyl phthalate (DBP) of purity 99.8 % was purchased from Loba Chemie. HPLC grade methanol (CH_3OH), acetonitrile (CH_3CN), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2) from Merck was used in analysis. Analytical grade chlorobenzene and ethyl acetate ($CH_3COOC_2H_5$) were used as purchased. Polystyrene divinyl benzene polymer (8 % cross linking beads) were a kind gift from Doshi Ion (Pvt) Ltd. 10 mL glass centrifuge tubes with flat bottom were fabricated to get conical shape bottom for DLLME. 10 µL Hamilton micro syringe was used for introduction of analytes after extraction and also as extraction medium for SDME. Centrifugation was performed on centrifuge machine Sigma 3K30 model.

Microcrystalline cellulose (MCC) prepared as discussed in chapter 2a was used as adsorbent for SPE. Analysis of the target analytes was performed on Shimadzu gas chromatograph – GC 2010 Plus equipped with an On column PTV injector and a flame ionization detector (FID). The chromatograms obtained were integrated by the GC solution software. The capillary column used is Rtx-5 with the dimensions 30m×0.53mm×5µm (length × i.d. × film thickness) and stationary phase as cross bonded 5% diphenyl and 95% dimethylpolysiloxane. The injection was performed in split less mode. The injector temperature was kept at 280 °C. The column flow rate was set at 3.89mL/min. The column temperature programming was as follows: the initial oven temperature was held at 200 °C for and held for 1 min, then raised to 240 °C at a rate of 25 °C for 3 mins. Further, it was raised to 270 °C at a heating rate of 15 °C and held for 3 mins and then finally at 275 °C at a rate of 5 °C and held for 5 mins. The FID temperature was maintained at 300 °C. The volume of

injected liquid samples was 1.0 µL. The total analysis time was 15 mins for this method.

3.3 Method

The used glassware was initially cleaned with chromic acid. It was then rinsed with water several times so that complete removal of chromic acid was achieved. In case if traces of acid remained, a dilute basic solution was added to remove it and finally rinsed again with water several times. Finally, the cleaned glassware was then rinsed with acetone and dried in oven at 100 °C for an hour.

3.3.1 Solid Phase Extraction

Solid phase extraction method was preferred to solvent extraction due to less consumption of organic solvents. Instead of employing the commonly used silica based reversed phase C8 or C18 SPE phases, we chose to use a polystyrene based SPE (PSDVB) phase. These were compared with other adsorbents as extraction medium.

Conditioning of PSDVB beads

10g of PSDVB beads were washed in a soxhlet extractor with 350 mL of methanol for 3 hours followed by 350 mL of water for 10 hours and again with 350 mL methanol for 3 hours. Then the beads were dried in vacuum oven at 50 °C for 3 hours. These treated beads were used for pre-concentration experiments [5].

Procedure:

Pre-concentration in SPE was carried out using a glass column with stopcock. The adsorbent material i.e., PSDVB beads, were packed in these columns. The columns were loaded with different amounts of the adsorbent material to achieve maximum extraction.

The extraction columns were washed with methanol (6 mL) and milli-Q water (6 mL). The aqueous samples of target analytes in the mixture were passed through the loaded column. Aqueous sample of the mixture containing the target analytes was prepared by diluting the stock solution of 500 µg mL^{-1}. This aqueous sample was passed through the columns for extraction. After the extraction the columns were allowed to drain off completely with the help of vacuum and dried with a flush of nitrogen gas for 20-25 mins. Elution was performed with 2mL of CH_3OH ***(figure 4)***. This solution was analyzed by GC by employing the instrumental method described

above. Experiments by varying the amount of adsorbent were performed following the extraction procedure. The methodology for the solid phase extraction method followed is described in the flow chart ***(figure 7)***.

Vortex assisted solid phase extraction was also used, 0.5 g PSDVB adsorbent was added in an aliquot of 10 mL spiked at a concentration of 1.0 µg mL^{-1} with the target analytes and vortexed for 5 mins in a test tube and then introduced with the adsorbent in the glass column and allowed the sample to pass through, then the adsorbent was dried under vacuum and the analytes were extracted with 2 mL of solvent (CH_3OH). The experiment exhibited poor recovery of all the analytes.

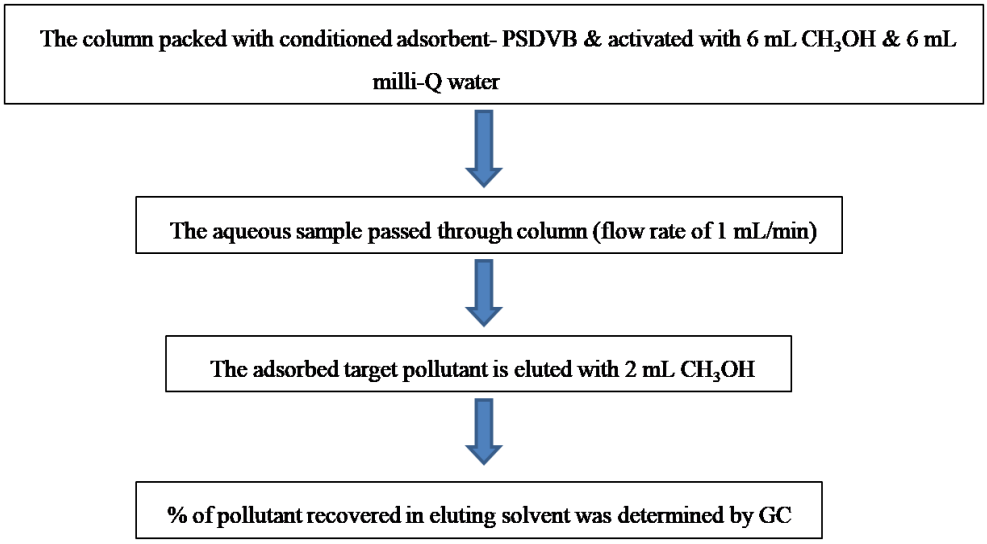


Figure 7 Flow chart for SPE steps

Stock solutions of the phthalates of concentration 100µg mL^{-1} were prepared in CH_3OH. Working standards with appropriate dilutions of the stock solution were prepared from 0.5 µg mL^{-1} to 10 µg mL^{-1}, to achieve calibration curves. The stock solutions and the diluted solutions were stored in glass volumetric flasks at 4 °C. Linear calibration curves were obtained for the phthalate esters.

3.3.2 Dispersive liquid liquid microextraction

A 5 mL aliquot of aqueous sample spiked with 1.0 µg mL^{-1} of target analytes was placed in a fabricated conical bottom centrifuge glass vial. To this a mixture of 0.8 mL acetonitrile (CH_3CN) and 70 µL dichloromethane (CH_2Cl_2) was added rapidly which resulted in a cloudy solution. After centrifugation for 6 minutes at 4500 rpm, the sedimented phase ***(figure 8)*** was taken out with the help of microsyringe and 1 µL of this solution was introduced in the GC system.

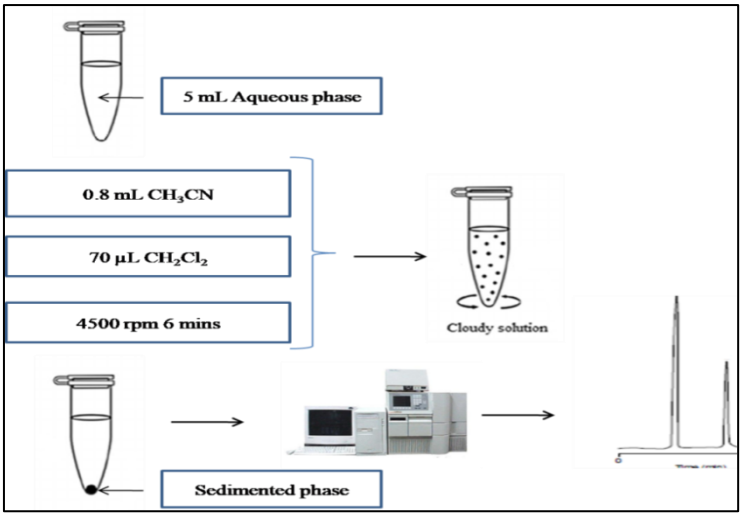


Figure 8 Method for DLLME

For the preparation of calibration curve stock solutions of the phthalates of 1000 µg mL^{-1}concentrationswere prepared in CH_2Cl_2. Working standards with appropriate dilutions of the stock solution were prepared from 1 µg mL^{-1} to 100 µg mL^{-1} (1, 5, 10, 20, 30, 40, 60, 80 and 100 µg mL^{-1}). The stock solutions and the diluted solutions were stored in glass volumetric flasks at 4°C.Linear calibration curves were obtained for the phthalate esters. Parameters such as effect of salt addition, centrifugation time, etc were studied based on one factor study at a time.

3.3.3 Single drop microextraction

A 10 mL aliquot of aqueous sample spiked with 1.0 µg mL^{-1} of target analytes was placed in a 15 mL glass vial. In this sample an upside down syringe was suspended containing the extraction solvent. The aqueous sample was stirred at 300 rpm and the extraction solvent droplet was suspended in presence of continuous stirring for 15 minutes. After extraction was complete, the extraction solvent droplet was retracted back in the Hamilton micro syringe. The syringe was cleaned without disturbing the syringe and then the extracted solvent was introduced in the GC system.

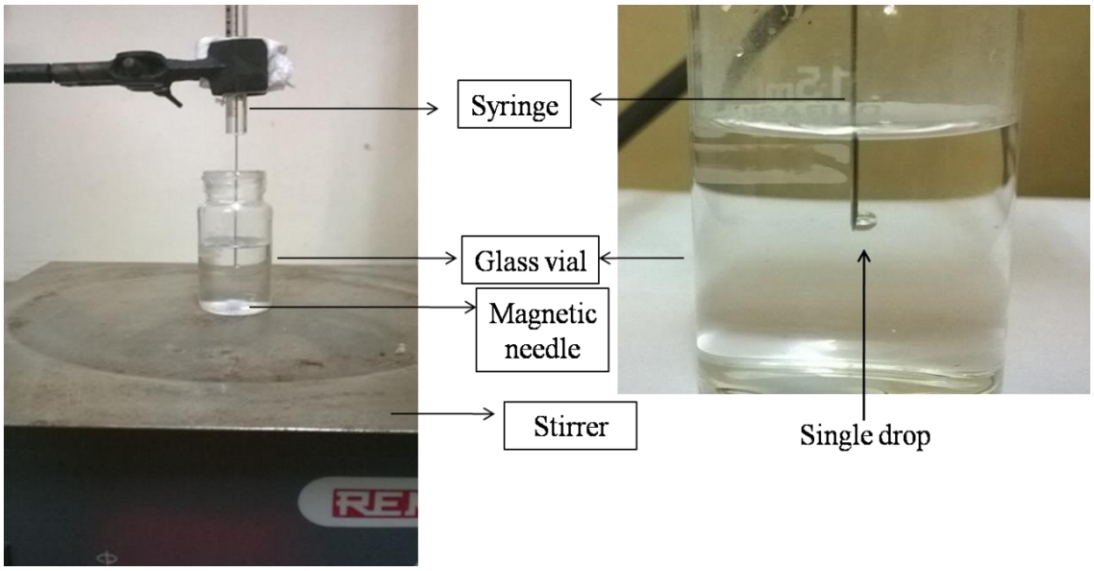


Figure 9 Picture from experimental setup for SDME in laboratory

For the preparation of calibration curve stock solutions of the phthalates of 1000 µg mL^{-1}concentrations were prepared in CH_3OH. Working standards with appropriate dilutions of the stock solution were prepared from 1 µg mL^{-1} to 100 µg mL^{-1} (1, 5, 10, 20, 30, 40, 60, 80 and 100 µg mL^{-1}). The stock solutions and the diluted solutions were stored in glass volumetric flasks at 4°C. Linear calibration curves were obtained for the phthalate esters. Parameters such as effect of solvent, salt addition, extraction time, etc were studied based on one factor at a time method at a time.

3.4 Results and discussion

3.4.1 Solid phase extraction

To determine the concentration of phthalate esters in real water samples, a method needed to be developed with the help of which the concentration of the phthalate esters extracted could be estimated. In general, the procedure followed is to develop calibration curve (or graph) for the analytes in methanol solvent. And then calculating the extraction efficiency as the ratio of concentration of analyte extracted over initial concentration taken (spiked) and computing it in terms of percentage. Before the analysis of phthalates was initiated the analytical method was validated in terms of statistical aspects such as:

(i) Linear dynamic range (LDR): The linear dynamic range was established by injecting five different concentrations of the standards of each analyte in triplicates in the Gas Chromatograph system. It was a plot of the intensity of peak

(mean of all the three injections) against concentration (µg/L). LDR helps establish the correlation coefficient (r), slope and the intercept.

(ii) Limit of detection (LOD): A key parameter for analytical data analysis. It is the lowest limit or amount of target analyte that can be detected but need not necessarily be quantitated [6]. The LOD was calculated as :

$$LOD = \frac{3.3 * \text{standard deviation of response } (\sigma)}{\text{The slope of calibration curve } (S)}$$

(iii)Limit of quantitation (LOQ): limit of Quantitation is defined as the lowest limit that can be reliably quantified for an analyte [6, 7]. The LOQ was calculated as :

$$LOQ = \frac{10 * \text{standard deviation of response } (\sigma)}{\text{The slope of calibration curve } (S)}$$

(iv) Precision (% RSD): A vital parameter in analysis, defined as the degree of achieving closeness to a same value determined for a set of duplicate measurements performed. It indicates the distribution of the value. The lesser the distribution, more is the precision ***(figure 10)***.

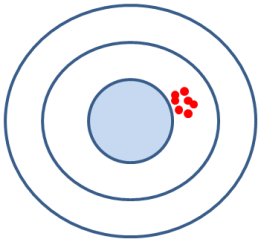


Figure 10 Figure depicting Precision

(v) Accuracy Accuracy is defined or expressed as the closeness of a value to a true or reference value (figure 11).

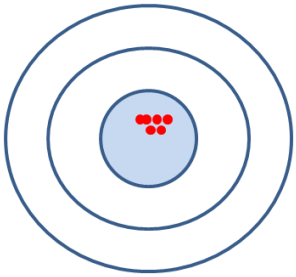


Figure 11 Figure depicting Accuracy

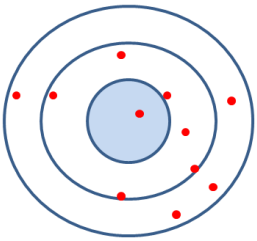


Figure 12 Accurate but not precise

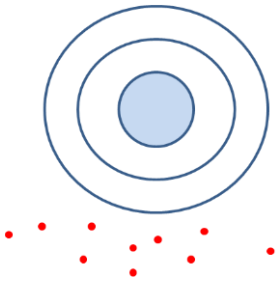


Figure 13 Neither accurate nor precise

Accuracy and precision (figure 12 and 13) are two important factors involved in performing statistical data analysis. Both express closeness to a value, accuracy refers to how close a value is to a true or reference value. And precision refers to the closeness of the values to each though they can be far from the true value (in figure 3.6).

(vi) Repeatability: The five replicate injections of single sample solution containing the target analytes along with sequential injections of standard solution were injected into GC using the selected parameters. % RSD was calculated and reported [8 and 9].

(vii) Coefficient of variance (%RSD): It is the ratio of standard deviation to the mean. The higher the coefficient of variance, the higher the level of dispersion around the mean. Precision is defined by it

$$\%\ RSD = \frac{Standard\ deviation}{Arithmetic\ mean} \times 100$$

(viii) % Recovery: It is the ratio of concentration achieved after extraction to its initial concentration. It is determined by the following formula:

$$\%\ Recovery = \frac{Concentration\ after\ extraction}{Initial\ concentration} \times 100$$

The correlation coefficient for linearity for concentration range 0.5 to 10.0 µg mL^{-1} was found to be 0.9993 for DMP and for the remaining analytes tabulated in table 1. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.08 and 0.27 µgmL^{-1}, respectively listed in table 2. The precisions (% RSD) and accuracies (%

Recovery) were found to be as tabulated in table 1 for DMP, DEP and DBP respectively.

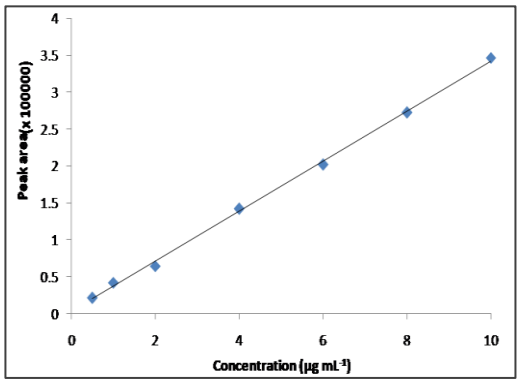


Figure 14 Calibration curve for DMP

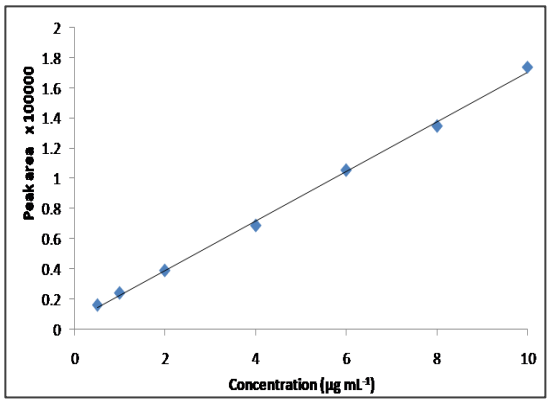


Figure 15 Calibration curve for DEP

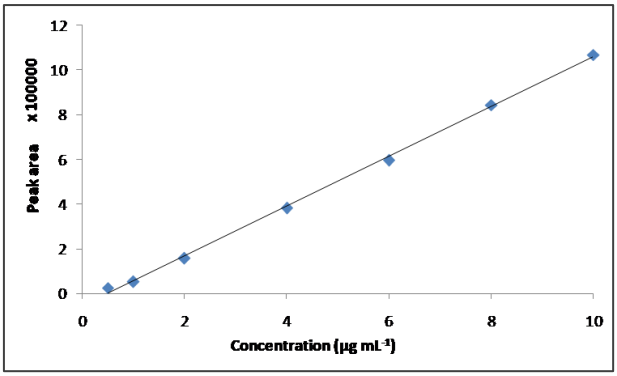


Figure 16 Calibration curve for DBP

The calibration curves have been prepared in methanol solvent (figure 14 to 16). Table 1 depicts the statistical data for all the three analytes.

Analyte	*X variable*	*Intercept*	*LDR: 0.5 to 10 µg mL^{-1} y = mx ± c*	*R^2*	*% RSD*
DMP	33956	2788	33956x + 2788	0.9986	4.26
DEP	16419	6269	16419x + 6269	0.9991	3.97
DBP	88498	- 1124	34105x - 1124	0.9994	3.55

Table 1 Statistical data for the three analytes (n=3)

Analyte	*LOD(µg mL^{-1})*	*LOQ(µg mL^{-1})*	*Recovery (%)*
DMP	0.08	0.27	96
DEP	0.12	0.40	52
DBP	0.26	0.80	25

Table 2 LOD and LOQ for all the analytes

To study the effect of adsorbents on the extraction recovery of target analytes, we have screened PSDVB (8%), Florisil, Microcrystalline cellulose MCC (commercial and prepared) as adsorbents for solid phase extraction of phthalate esters. 10 mL 1.0 µg mL^{-1} sample containing the target analytes was passed through the column containing the adsorbents respectively at a flow rate of 1.0 mL/min. Then, dried under vacuum and eluted with 2 mL CH_3OH, of which 1.0 mL was introduced in GC. The purpose of the study was to screen the best adsorbent with which maximum % recovery of the analytes could be achieved ***(table 3)***.

Adsorbents (0.5 g)	*DMP*	*DEP*	*DBP*
		% Recovery	
Florisil	89.50	55.00	12.00
Commercial MCC	9.50	10.60	3.50
Prepared MCC	3.60	5.00	4.30
(1:1) CMCC + Florisil	64.90	5.36	3.52
Vortex assisted SPE	2.50	28.40	9.23
PSDVB 8%	**93.90**	**48.54**	**23.12**

Table 3 Change in adsorbents studied for SPE (n=3)

We assumed that by using vortex assisted solid phase extraction; we would achieve higher % recovery of analytes. As the adsorbent is vortexed with an aliquot of sample solution containing analytes. The analytes were extracted with 2 mL of solvent (CH_3OH). The experiment exhibited poor recovery of all the analytes.

Effect of amount of adsorbent:

To study the effect of amount of adsorbent on extraction of target analytes, the amount of 8% polystyrene divinyl benzene (PSDVB) was varied from 0.25 to 1.0 g of the adsorbent. An aliquot of aqueous sample containing the target analytes spiked at a concentration of 1.0 µg mL^{-1} were passed through the column at a flow rate of 1.0 mL min^{-1} with the adsorbed already packed in the column and conditioned with methanol and water ***(table 4 and 5)***.

8% PSDVB	*Average Peak area from GC*		
(in g)	*DMP*	*DEP*	*DBP*
0.25	33416	13812	5215
0.50	34672	17238	8164
1.00	35929	16284	10164

Table 4 Average peak area for analytes from GC

8% PSDVB (in g)	*(% R) DMP*	*(% R) DEP*	*(% R) DBP*
0.25	90.20	45.94	18.59
0.50	93.90	54.62	27.23
1.00	97.60	66.80	39.30

Table 5 Effect of change in amount of adsorbent (n=3) on % recovery of PAE

From the experiment and its analysis performed by GC, it was observed (table 4) that 1.00 g was the amount of adsorbent for SPE experiments, at which maximum extraction (% Recovery) of the analytes was achieved, which was observed as signal in the chromatogram exhibited in figure 17.

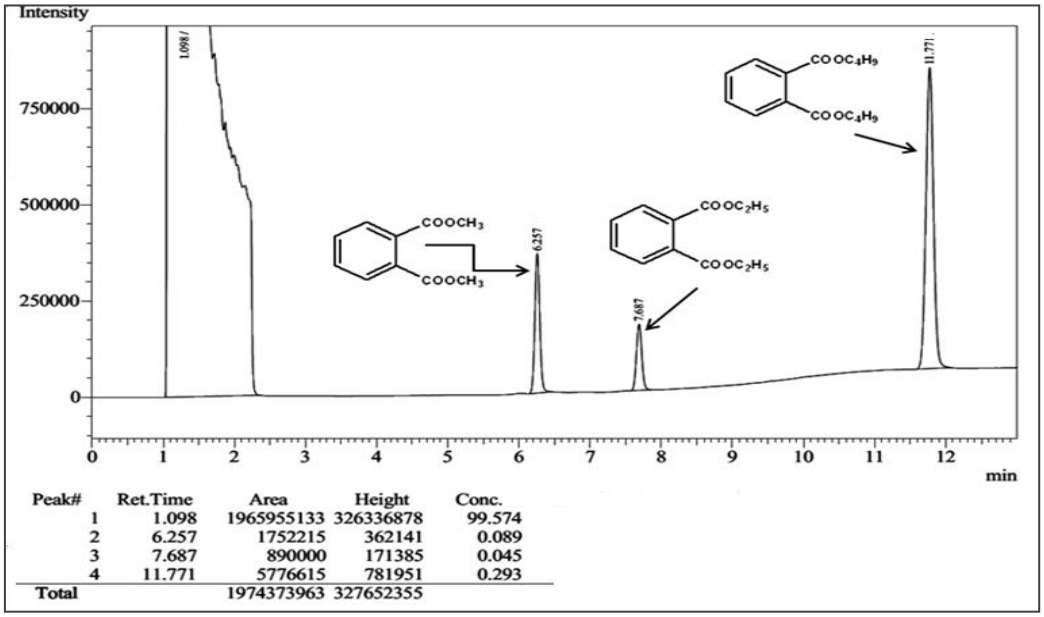


Figure 17 Representative chromatogram for the target analytes

Further, effect of changing volume of aqueous sample was studied. Volume of aqueous sample was varied from 10 to 60 mL by keeping the concentration constant of target analytes (1.0 µg mL^{-1}) and amount of adsorbent 1.0 g. It was observed (figure 3.14) that with increase in volume, the extraction of the analytes increased and remained constant after 40 mL. This may be due to saturation of the adsorbing

efficiency of the adsorbent. Therefore, sample volume of 40 mL was kept constant during analysis.

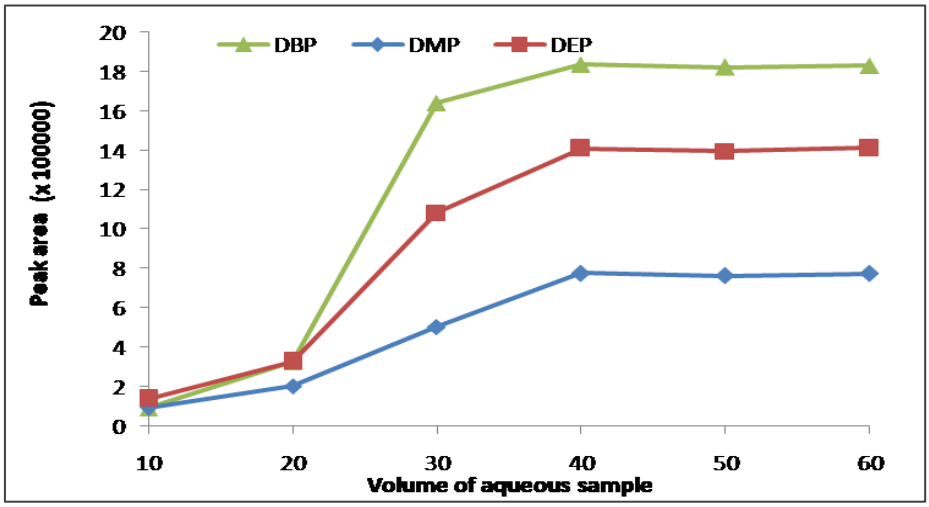


Figure 18 Effect of volume of aqueous sample (n=3)

With these optimized parameters, the extraction of the target analytes spiked at concentrations of 0.5, 1.25, 2.5 and 5.0 µg mL^{-1} was observed and the pre-concentration factor increased as the concentration increased ***(figure 19)***.

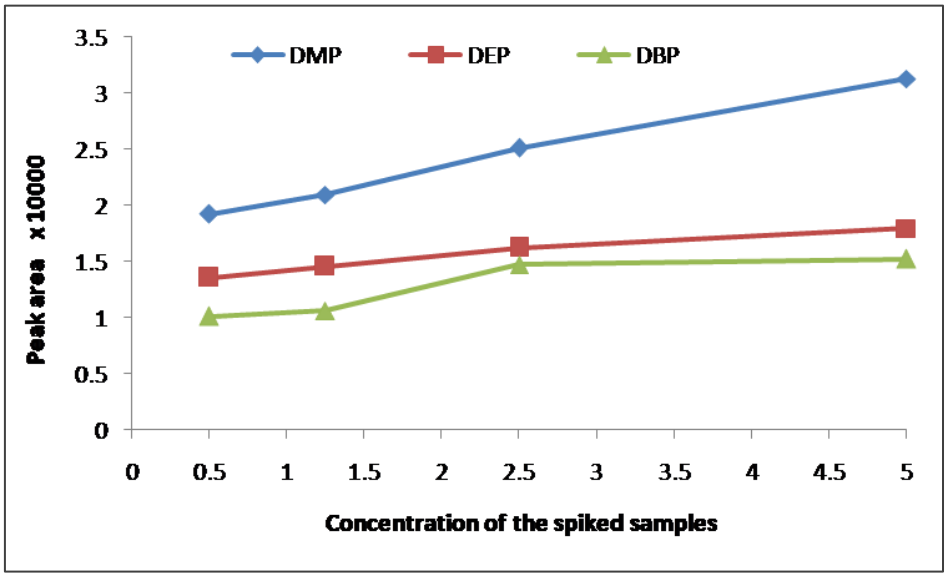


Figure 19 Concentration achieved after extraction

Thus, from the parameters optimized, 1.00 g of Florisil and PSDVB exhibited high extraction recoveries of the analytes respectively. Therefore, we chose 1.00 g of PSDVB as adsorbent for our experiments further which was subjected for SPE with a 40 mL volume of aqueous sample containing target analytes at a flow rate of 1.0 mL/min.

3.4.2 Dispersive liquid liquid microextraction

To begin with the method a calibration curve was needed to be established so that the concentration of phthalate esters from real water samples could be determined. This study was helpful in calculating the extraction efficiency as the ratio of concentration of analyte extracted over initial concentration taken (spiked) and computing it in terms of percentage. The method was optimized based on five parameters (LDR, LOD, LOQ, %RSD).

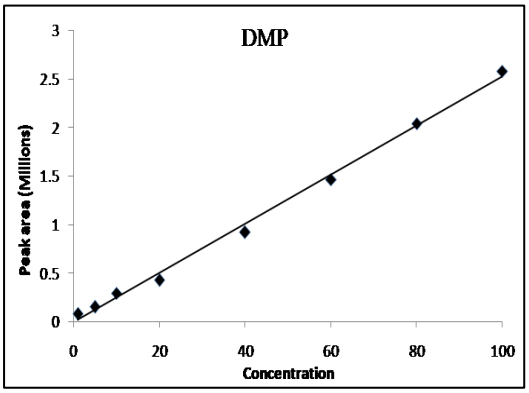


Figure 20 Calibration curve for DMP

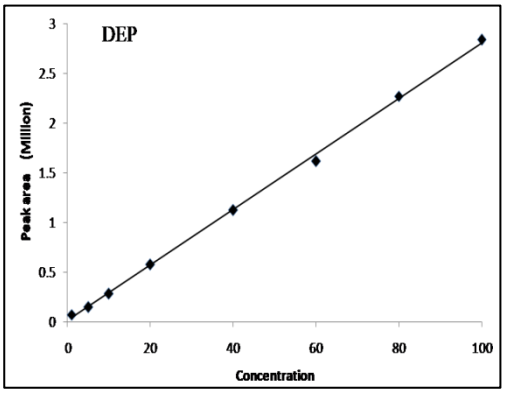


Figure 21 Calibration curve for DEP

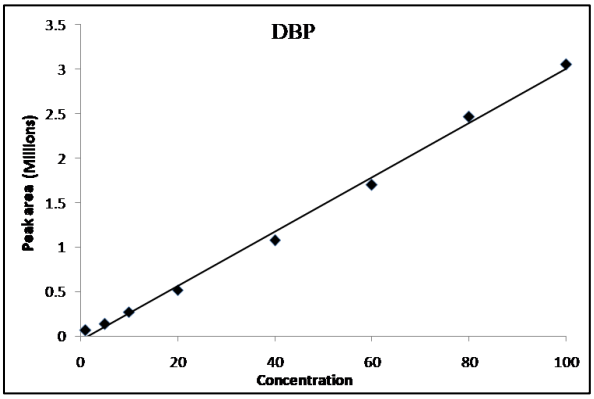


Figure 22 Calibration curve for DBP

The correlation coefficient for linearity for concentration range (LDR), the limit of detection (LOD) and limit of quantitation (LOQ) were 0.15 and 0.46 $\mu g mL^{-1}$, respectively for dimethyl phthalate tabulated in table 6 and 7. Enrichment factors for all the analytes by the optimized parameters were tabulated in table 7.

Analyte	*X variable*	*Intercept*	*LDR: 1 to 100 µg mL^{-1} $y = mx \pm c$*	*R^2*	*% RSD (n=3)*
DMP	25319	- 8011	y = 25319 x- 8011	0.9995	3.15
DEP	27974	9307	y = 27974 x + 9307	0.9988	2.21
DBP	30524	- 45108	y = 30524 x- 45108	0.9969	2.67

Table 6 Statistical data for target analytes

Analyte	*LOD*	*LOQ*	*Enrichment Factor (EF)*
DMP	0.07	0.23	90
DEP	0.09	0.28	91
DBP	0.16	0.49	96

Table 7 LOD and LOQ for target analytes

Further, one factor at a time method of observing the effect of various experimental parameters was studied. The parameter studied and the optimized ones are listed in table 8.

Parameters studied	*Range*	*Optimized parameter*
Effect of extraction solvent	CH_2Cl_2, $CHCl_3$, Chlorobenzene	CH_2Cl_2
Effect of disperser solvent	CH_3OH, CH_3CN, CH_3COCH_3	CH_3CN
Centrifugation speed	2000 to 5000 rpm	4500 rpm
Centrifugation time	3 to 6 minutes	6 minutes
Effect of salt addition	0 to 10 % (w/v)	2.50 %

Table 8 Parameters studied and optimized

For DLLME experiments, a mixture of extraction solvent and disperser solvent is introduced in the aqueous sample rapidly. We began our initial study by studying the combinations of the mixture to observe the behaviour of solvent mixture sedimentation at the bottom of the conical bottom centrifuge vial. In a conical bottom centrifuge vial containing 5 mL aliquot of water sample spiked with the three target analytes at a concentration of 1 µg mL^{-1}, the extraction solvent and disperser solvent mixture was introduced rapidly with the help of a 5 mL (capacity) glass syringe that resulted in the formation of a cloudy solution and then centrifuged for 3 minutes at 5000 rpm. 1 µL of the sedimented extraction solvent was retracted with the help of a Hamilton micro syringe. The syringe was cleaned with a tissue paper and then introduced in the GC system for analysis. Table 8 exhibits the observation for sedimentation of the mixture in the aqueous sample.

Extraction recoveries were calculated for each case (combination) and amongst them with CH_3CN as disperser solvent and extraction solvent as $CHCl_3$ and CH_2Cl_2 higher recoveries were obtained. Thus, a study was done with combination of CH_3CN and CH_2Cl_2 and that of CH_3CN and $CHCl_3$ mixture.

S.No.	Dispersing solvent		Extracting solvent	Observation (Sedimentation)
1	0.2mL CH_3OH	a.	75µL CH_2Cl_2	No
		b.	75µL $CHCl_3$	Yes
		c.	75µL Chlorobenzene	Yes
2	0.2mL CH_3CN	a.	75µL CH_2Cl_2	Yes
		b.	75µL $CHCl_3$	Yes
		c.	75µL Chlorobenzene	Yes
3	0.2mL CH_3COCH_3	a.	75µL CH_2Cl_2	No
		b.	75µL $CHCl_3$	Yes
		c.	75µL Chlorobenzene	Yes
4	0.2mL ethyl acetate	a.	75µL CH_2Cl_2	Yes
		b.	75µL $CHCl_3$	Yes
		c.	75µL Chlorobenzene	Yes but more centrifugation time needed.
5	0.1mL CH_3OH + 0.1mL CH_3CN	a.	75µL CH_2Cl_2	No
		b.	75µL $CHCl_3$	Yes
		c.	75µL Chlorobenzene	Yes

Table 9 Study on combination of E.S + D.S

The enrichment factors were calculated as the ratio of concentration calculated from calibration graph over concentration of analytes taken (spiked) as shown in table 9. The combination of acetonitrile and dichloromethane exhibited higher enrichment factors.

$$Enrichment\ factor = \frac{Concnetration\ calculated\ from\ calibration\ graph}{Calculation\ of\ analytes\ taken\ (spiked)}$$

EF	0.2 mL ACN		0.3 mL ACN	
	A	B	A	B
DMP	10.40	6.94	14.37	10.66
DEP	10.64	6.09	14.15	10.39
DBP	12.93	8.25	15.23	11.17

Table 10 Enrichment factor studied (A = CH_2Cl_2 and B = $CHCl_3$)

Thus, dichloromethane as extraction solvent and acetonitrile as disperser solvent exhibited higher enrichment factor for our target analytes compared to chloroform.

Effect of extraction solvent:

From the initial studies of sedimentation performed, organic immiscible solvent such as $CHCl_3$, CH_2Cl_2 and chlorobenzene were studied as extraction solvent. Thus, an aliquot of 5 mL aqueous sample containing the target analytes spiked at a concentration of 1 µg mL^{-1} was studied. Disperser solvent was 0.3 mL acetonitrile as data obtained from the initial experiments and 75 µL of extraction solvent.

Analytes	*CH_2Cl_2*	*$CHCl_3$*	*Chlorobenzene*
	Average Peak area from GC (n=3)		
DMP	1635001	735588	645741
DEP	2689314	938663	922526
DBP	5905837	1552386	1392523

Table 11 Average peak area for analytes

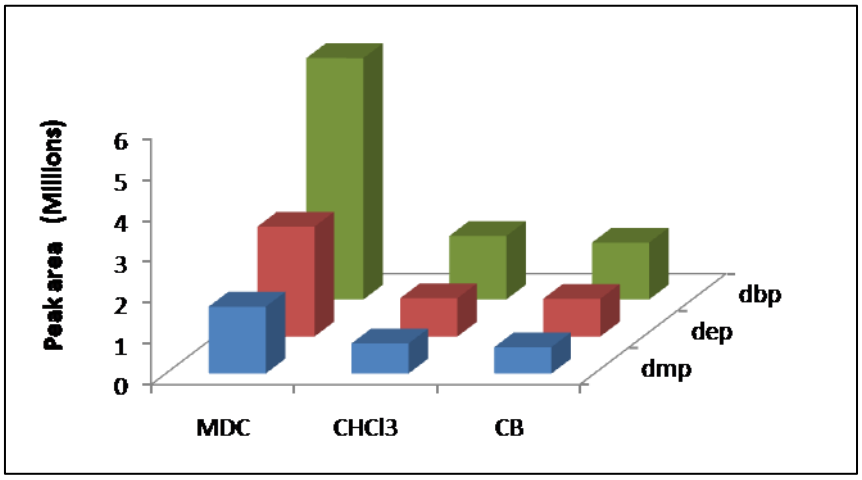


Figure 23 Effect of extraction solvent

Highest extraction recovery (or enrichment factor) was achieved with CH_2Cl_2 (MDC) as extraction solvent ***(figure 23)***.

Effect of volume of disperser solvent:

The role of disperser solvent is critical as it needs to fulfil the criteria of having solubility both in water and the organic phase. This serves as an advantage in extraction as it helps in forming a cloudy solution (an emulsion) which results in dispersion of fine droplets of extraction solvent providing larger interface for the analytes to get extracted. Thus, its volume should be optimum so that with lower volume higher extraction recoveries and proper sedimentation of the extraction solvent are achieved. The effect of disperser solvent was studied with a 5 mL aliquot of sample spiked at a concentration of 1.0 µg $mL^{-1}$of the three target analytes. 50 µL of CH_2Cl_2 as extraction solvent was introduced in combination with different ratios

(volume) of acetonitrile (CH_3CN) rapidly with the help of glass syringe and it was observed that as the volume of the disperser solvent increased the concentration of the target analytes extracted also increased.

There was a gradual increase in concentration of the extracted target analytes as the volume of the disperser solvent increased till 0.8 mL and beyond that it got constant, at 1.0 mL slight increase was observed and beyond that the extraction recovery decreased with increase in volume. This may be due to the solubility of phthalate esters increasing in acetonitrile – water solution and thus the extraction recovery decreasing with the increase in volume. Thus, 0.8 mL was chosen as the volume of disperser solvent, table 12.

Vol of D.S	***DMP***	***DEP***	***DBP***
	Average Peak area from GC (n=3)		
0.20	691820	798677	1163934
0.30	845035	894078	983772
0.40	960377	1075789	1189738
0.50	1171582	1329369	1448599
0.60	1586895	1837665	2047944
0.70	1894359	2329799	3079198
0.80	2886007	4186861	5581012
0.90	2735645	4325654	5575612
1.00	2628825	4537813	5685397
1.10	2465423	4263543	4965321
1.20	1517103	3985642	4138868

Table 12 Average peak area for analytes

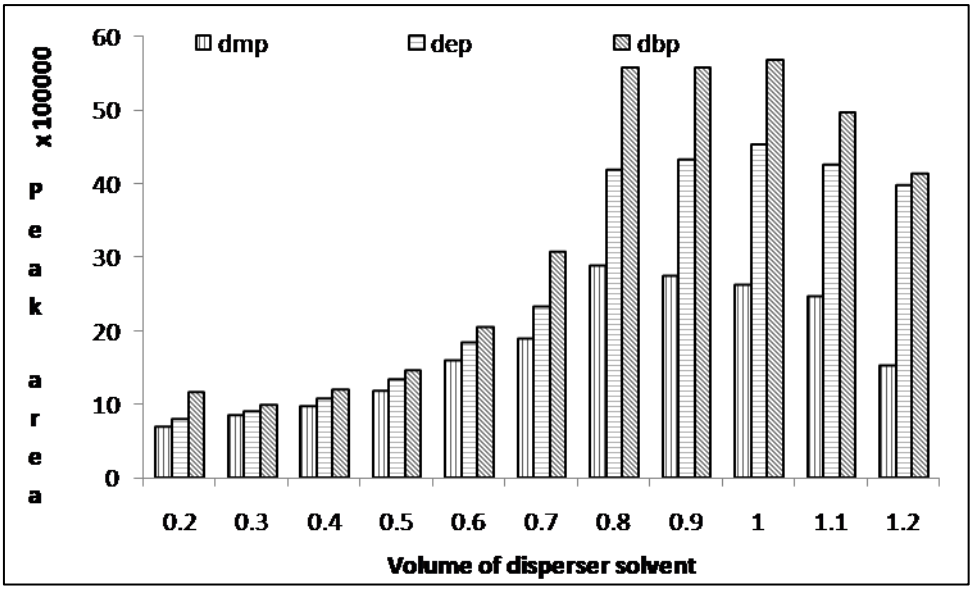


Figure 24 Effect of volume of disperser solvent (n=3)

Effect of volume of extraction solvent:

With the optimized conditions so far, the effect of volume of extraction solvent was studied to determine at which concentration we get maximum extraction recovery (or enrichment factor). A mixture of 0.8 mL acetonitrile as disperser solvent and CH_2Cl_2 as extraction solvent with different volumes (65, 70, 75, 80 and 85 µL) was injected rapidly in 5 mL of an aqueous sample containing the target analytes spiked at a concentration of 1.0 µg mL^{-1} and centrifuged at 5000 rpm for 3 mins. 1µL of the sedimented phase was collected through a Hamilton micro syringe and the extracted solution was introduced in the GC system for analysis.

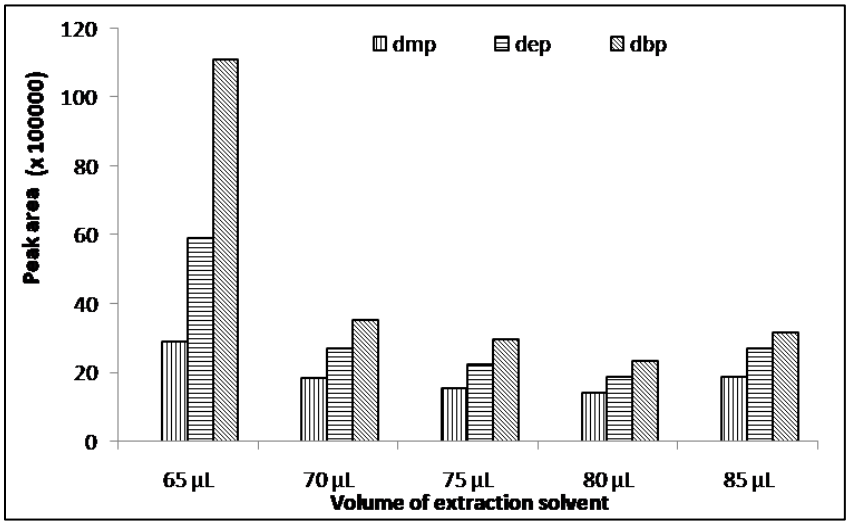


Figure 25 Effect of volume of extraction solvent (n=3)

ES volume (µL)	*Average peak area (n=3)*	*Volume of sedimented phase (µL)*
65	2926698	3 to 5
70	1871136	15 ± 5
75	1557665	15
80	1424562	30
85	1891995	30

Table 13 Sedimented phase observed after extraction

It was observed that maximum extraction was achieved with 65 µL of extraction solvent ***(figure 25)***, but the volume of the sedimented phase was very low (3 to 5 µL) ***(table 13)***. With repeated number of experiments with this combination we found difficulty in collection of this low volume of sedimented phase and also in few experiments it was below 3 µL, taking into account the aspects of repeatability, droplet volume, and enrichment factor. 70 µL of extraction solvent was chosen as the extraction volume.

Effect of centrifugation time:

Centrifugation time is the time for which the ternary solvent mixture (aqueous phase containing the target analytes + disperser solvent + extraction solvent) is centrifuged. A 5 mL of aqueous sample spiked with target analytes at 1.0 µg mL^{-1} concentration was used. To this, a mixture of 70 µL of CH_2Cl_2 and 0.8 mL of CH_3CN was rapidly injected and centrifuged for 3, 4, 5 and 6 minutes at 5000 rpm.

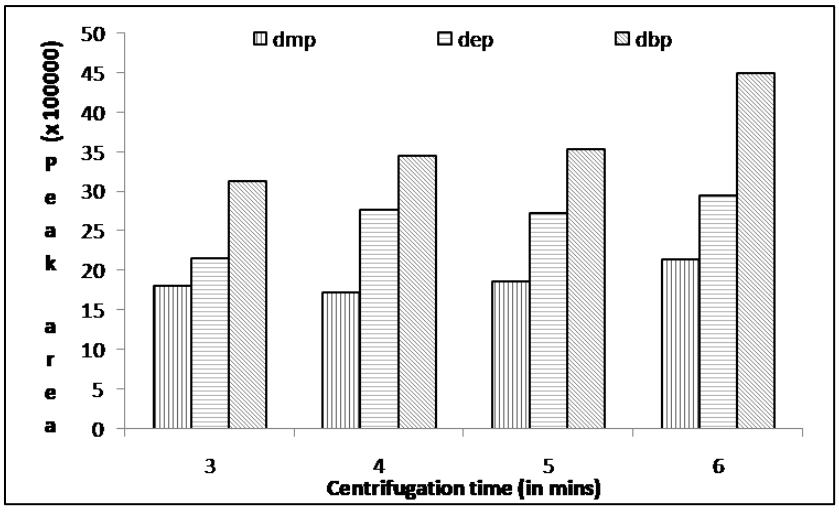


Table 14 Effect of centrifugation time on extraction of target analytes

Centrifugation helps in acceleration of phase separation [11] and at 6 minutes highest extraction was achieved, though it did not affect the volume of the sedimented phase ***(figure 14)*** and enrichment factors are tabulated in table 16.

Time (mins)	DMP	DEP	DBP
	Average peak area (n=3)		
3	1811071	2166700	3140723
4	1722215	2766499	3452645
5	1871136	2731702	3542332
6	2146179	2960495	4497402

Table 15 Peak are for target analytes

Time (mins)	Enrichment Factor		
	DMP	DEP	DBP
3	35.92	38.56	52.18
4	34.16	49.28	57.29
5	37.10	48.65	58.76
6	42.54	52.74	74.40

Table 16 Enrichment factor for effect of centrifugation time

Thus, at 6 minutes higher enrichment factor for the three target analytes were achieved and chosen as the time for extraction.

Effect of centrifugation speed:

With the optimized parameters so far, the effect of centrifugation speed on extraction was observed. To a 5 mL aqueous sample containing target analytes spiked at 1.0 µg mL^{-1} concentration, 0.8 mL acetonitrile (CH_3CN) and 70 µL dichloromethane (CH_2Cl_2) were added rapidly and centrifuged for 6 minutes at different rpm.

The effect of centrifugation speed was monitored from 2000 to 5000 rpm (revolutions per minute) and average peak area for all the analytes are tabulated in table 17.

Centrifugation speed	DMP	DEP	DBP
	Average peak area (n=3)		
2000	1470558	1725801	1958655
2500	1911041	2291925	2919450
3000	1894359	2087389	2720097
3500	2309246	2933064	3568390
4000	2695074	3403282	3852552
4500	3549240	4534287	4703873
5000	2740869	3781744	3770389

Table 17 Effect of centrifugation speed

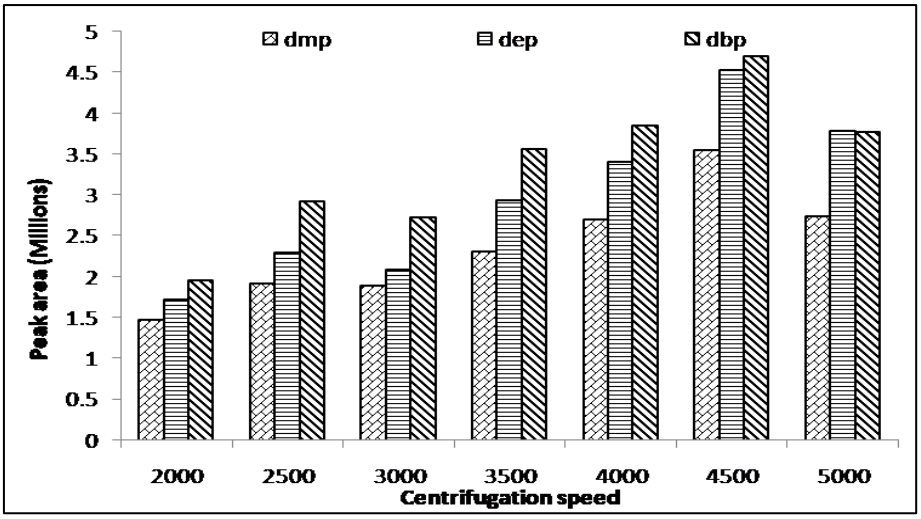


Figure 26 Effect of centrifugation speed on extraction

It was observed ***(figure 26)*** that with increase in centrifugation speed, extraction recovery (or enrichment factor) of the analytes increased. This may be due to the fact that acceleration helps in phase separation eliminating emulsification, thus facilitating extraction of the analytes. Thus, at 4500 rpm maximum extraction was achieved. But at 5000 rpm extraction efficiency reduced because higher speed further resulted in inaccurate phase separation.

Effect of salt addition:

Salt addition or salting out effect assists in extraction of analytes as salt reduces their solubility in aqueous phase. Salt effect was studied on extraction efficiency at different amounts from 0 to 10 % (w/v). Analytes spiked at a concentration of 1.0 µg mL^{-1} and with other optimized parameters so far, the experiments were conducted.

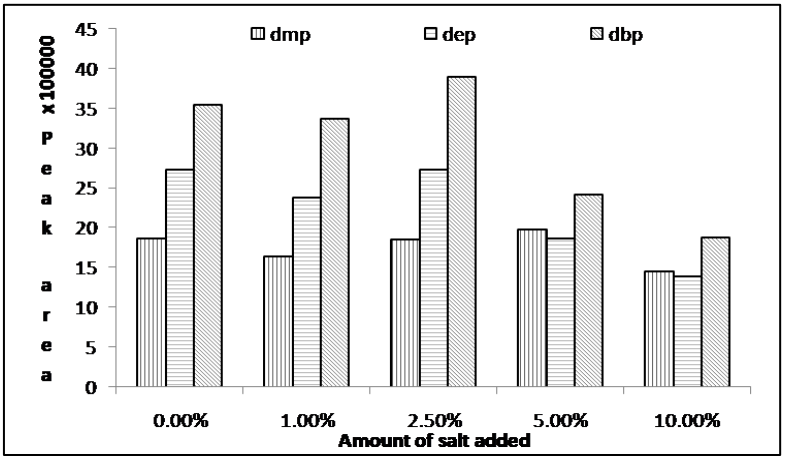


Figure 27 Effect of salt addition on extraction

It was observed that with increase in addition of salt at 2.5 % w/v, extraction efficiency increased. But with further increase in salt, extraction efficiency decreased. This could be because of increase in viscosity of aqueous phase [12]. This led to a decrease in the diffusion coefficient of analytes and thus, 2.5 % was chosen as the amount of salt added to the aqueous phase so that analytes are extracted to the maximum efficiency ***(figure 27)***.

Salt added (w/v)	***0.0%***	***1.0%***	***2.5%***	***5.0%***	***10.0%***
DMP	37.10	32.72	36.80	39.33	28.91
DEP	48.65	42.48	48.80	33.29	24.83
DBP	58.76	55.96	64.65	40.43	31.51

Table 18 Enrichment factor of analytes on addition of salt (n=3)

Effect of dispersing times:

The total mixture of extraction solvent and disperser solvent (70 µL + 0.8 mL) was injected directly in one go in the first case (mixture in whole part). In the second case, the total mixture was divided into two parts and then injected. In the third case, the total mixture of extraction and disperser solvent was divided into three parts and then injected. This study was conducted with a view to observe change in the extraction efficiency ***(table 19)***. The study was based on use of air as dispersant solvent. It was observed that this experiment had an increasing impact on extraction efficiency ***(figure 28)***.

Analytes	***1 part***	***2 parts***	***3parts***
DMP	2152442	3366369	5663087
DEP	2309406	4056511	6894457
DBP	3827219	5744825	8598542

Table 19 Average peak area for analytes (n=3)

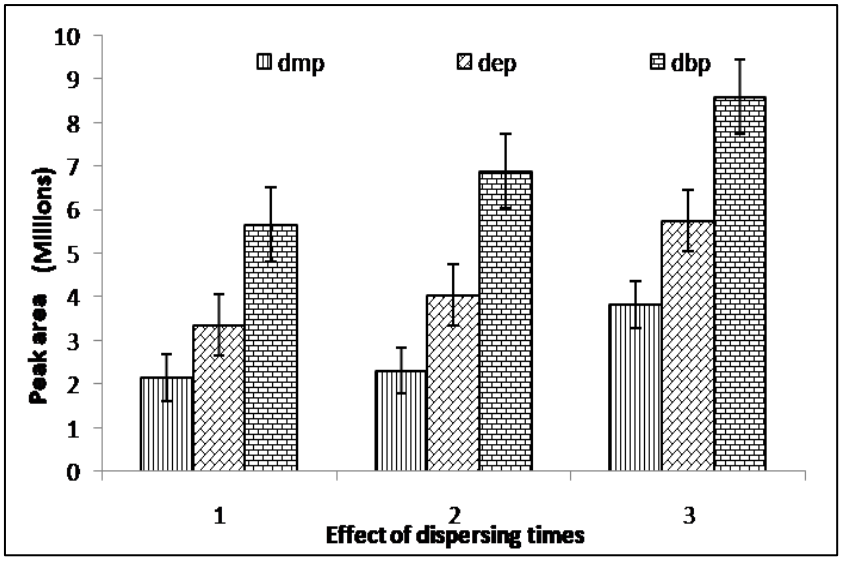


Figure 28 Effect of dispersing time on extraction efficiency

The increase in the extraction efficiency was due to the similar dispersing effect that was created when air had been used as dispersing agent. With all the optimized parameters, extraction of target analytes in spiked water, its repeatability and extraction in real water samples was studied.

3.4.3 Single drop microextraction

Calibration curve for ***(figure 29 to 31)*** the determination of phthalate esters was established by preparing appropriate dilutions of the stock solution in toluene. The calculation of phthalate esters in unknown real sample are calculated from these calibration graphs. The method was optimized with these parameters (LDR, LOD, LOQ, %RSD).

The correlation coefficient for linearity for concentration range (LDR), precisions (% RSD), the limit of detection (LOD) and limit of quantitation (LOQ) were 0.04 and 0.14 μg mL^{-1}, respectively and Enrichment factor were found to be as tabulated in table 20 and 21.

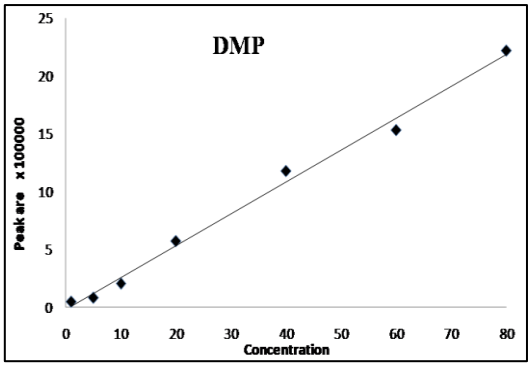


Figure 29 Calibration curve for DEP

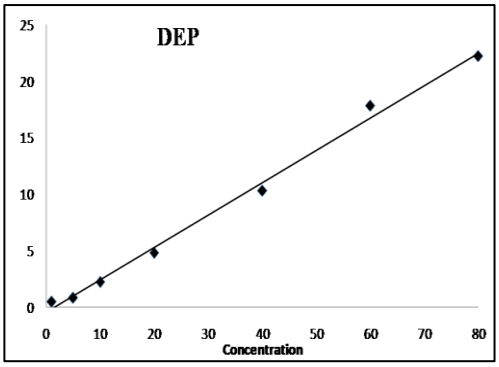


Figure 30 Calibration curve for DEP

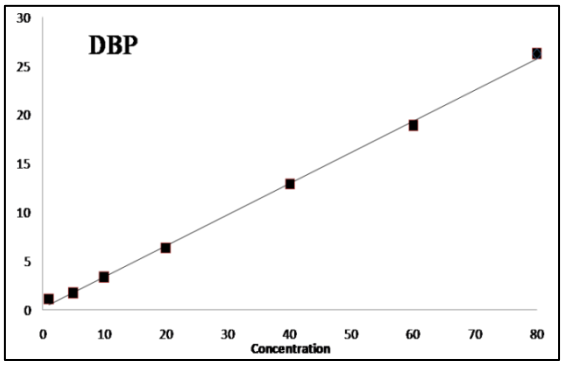


Figure 31 Calibration curve for DBP

Analyte	*X variable*	*Intercept*	*LDR: 1 to 100 µg mL^{-1} y = mx ± c*	*R^2*	*% RSD (n=3)*
DMP	27541	-16002.5	y = 27541 x- 16002.5	0.9932	2.08
DEP	28718	-47130	y = 28718 x - 47130	0.9968	3.48
DBP	31904	21984	y = 31904 x- 21984	0.9989	2.74

Table 20 Statistical data for target analytes

Analytes	*LOD*	*LOQ*	*Enrichment Factor*
DMP	0.04	0.14	92
DEP	0.03	0.10	138
DBP	0.03	0.11	152

Table 21 Statistical data 2 for analytes

An overview of the parameters studied and optimized for the extraction technique SDME have been tabulated in table 22.

Parameters studied	*Range*	*Optimized parameter*
Effect of extraction solvent	$CHCl_3$, Chlorobenzene, toluene, cyclohexane	Toluene
Effect of aqueous sample	5 mL to 20 mL	10 mL
Stirring rate	100 to 300 rpm	300 rpm
Extraction drop volume	0.7 to 1.0 µL	1.0 µL
Effect of salt addition	0 to 10 % (w/v)	0 %
Temperature (°C)	20 , 25, 30, 35, 40, 45	30

Table 22 Overview of parameters studied and optimized

Effect of extraction solvent:

Choice of extraction solvent plays a critical role in this technique, as the technique is dependent on the performance of the extraction solvent. The extraction is the medium in which the analytes diffuse into from aqueous solution. The solvent studied needs to be water immiscible and their chemical properties are listed in table 23 and the observation of drop persistence from initial experiments.

Solvent	*Observation – drop persists*	*Boiling point (°C)*	*Viscosity (cP)*	*Surface tension (dyn cm^{-1})*	*Water solubility (mg L^{-1}, 25 °C)*
n-hexane	✓	68.7	0.08	17.91	9.5
Cyclohexane	✓	80.7	1.0	-	55
Toluene	✓	110.6	0.59	28.53	526
Chlorobenzene	✓	132	7.68	33	200
Chloroform	✓	61	0.54	26.7	809
Dichloromethane	×	40	0.44	28.12	1.3×10^4

Table 23 Properties and drop persistence in SDME of extraction solvents tested

With preliminary experiments, the study of drop persistence (1 µL) was observed with stirring effect of 300 rpm for an extraction time of 10 minutes. It was observed that with all the solvents the droplet persisted for the minimum period of 10 minutes except for dichloromethane. In this case, the droplet persisted only for 3 – 4 minutes. In all the other cases, the respective droplet persisted for minimum 10 minutes. Thus, based on this the solvents except dichloromethane were used in this study.

10 mL aqueous sample spiked with the target analytes at a concentration of 1.0 µg mL^{-1} were extracted with the extraction solvent (1 µL) suspended from the micro syringe. A mixture of extraction solvents 100 µL of chloroform (c) and 100 µL of toluene (t) was prepared and from that 1 µL of mixture (c + t) was injected. Figure 32 exhibits the effect on extraction with the change in solvent. The purpose of a mixture of extraction solvent was to determine whether there is an increase in extraction efficiency.

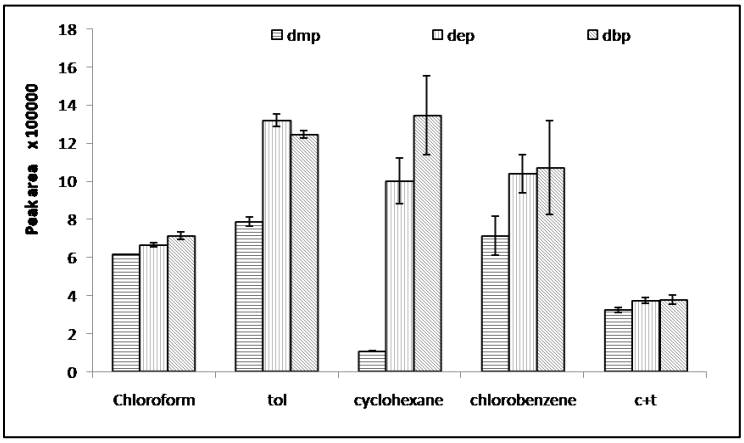


Figure 32 Effect of extraction solvent

Cyclohexane also exhibited higher extraction efficiency for diethyl phthalate and di-*n*-butyl phthalate but the droplet stability was an issue as it dislodged. Therefore, toluene was chosen as the extraction solvent for all the experiments as it exhibited maximum extraction for all the analytes.

Analytes	***Average peak area (n=3)***				
	$CHCl_3$	Toluene	Cyclohexane	Chlorobenzene	$CHCl_3$+ toluene
DMP	620332	788016	109230	714863	325376
DEP	667597	1322342	1003756	1039659	375915
DBP	713469	1245629	1346697	1071983	379499

Table 24 Average peak area for all the analytes achieved after extraction

Effect of stirring rate:

The preliminary permutation and combination of experiments with extraction solvent's behaviour at high stirring rate. The study was conducted at stirring rates of 100, 200 and 300 rpm ***(table 24)***.

Stirring rate	*DMP*	*DEP*	*DBP*
	Average peak area in GC (n=3)		
100	233087	288078	311556
200	316049	414060	442399
300	395339	569579	598770

Table 25 Effect of stirring rate (average peak area)

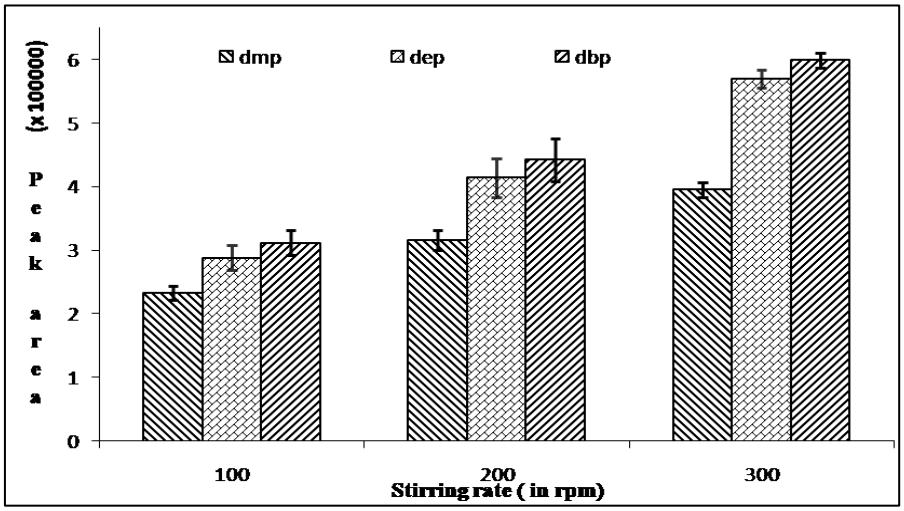


Figure 33 Effect of stirring rate on extraction of analytes

It was observed that as the rate of stirring increased, the extraction of analytes increased because of higher diffusion of target analytes in the sample assisting in achieving equilibrium rapidly between the aqueous and organic phase. The stirring rate could be increased further but the issue arises as the drop became unstable at higher speed and dislodged from the tip of syringe at higher stirring rate. Thus, stirring rate of 300 rpm was finalised and used in all experiments further.

Effect of extraction droplet volume:

This is the volume at which the target analytes are extracted from the aqueous sample using the microsyringe. The experiments were conducted with 10 mL aqueous sample spiked with 1.0 µg mL^{-1} of analytes stirred at 300 rpm.

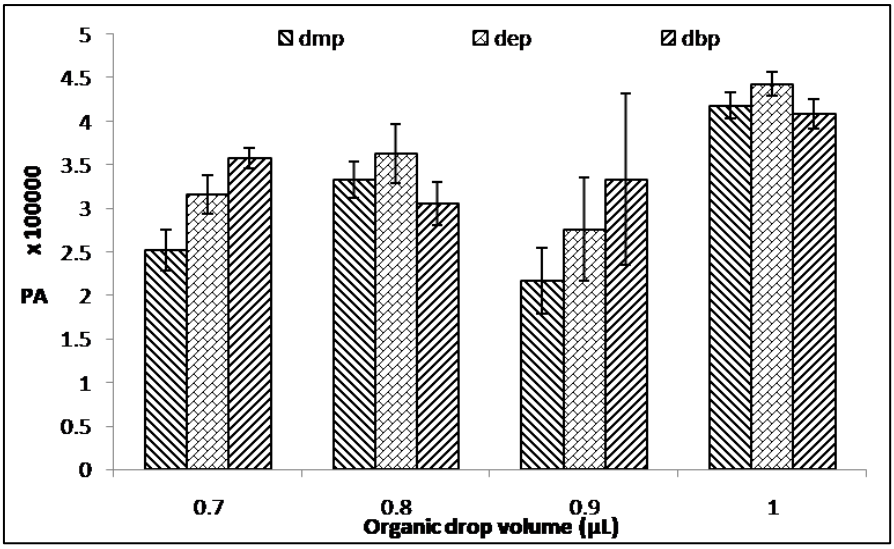


Figure 34 Effect of extraction droplet volume on extraction

All the parameters optimized so far were kept constant and only the droplet volume varied from 0.7 to 1.0 µL. Higher extraction recovery was achieved with 1 µL extraction droplet volume, it increased with the increase in droplet volume. But at the same time as the droplet volume increased, the issue of repeatability was compromised as the droplet became unstable, average peak areas for all the analytes are shown in table 26.

Organic drop volume (µL)	***DMP***	***DEP***	***DBP***
	Average peak area in GC (n=3)		
0.7	252095	315602	357741
0.8	332357	362684	305284
0.9	216310	276110	333612
1.0	417919	443035	408081

Table 26 Effect of organic drop volume

Effect of time parameter:

Effect of extraction time from 2.5 to 20 minutes was studied in order to achieve maximum extraction of analytes. Fortified samples at a concentration of 1.0 µg $mL^{-1}$with target analytes was extracted with 1.0 µL of extraction solvent (toluene) at 300 rpm. All the experiments were performed in triplicates for each extraction time. Highest extraction for all the three analytes was achieved at 15 minutes (figure 3.36). As time for extraction increased, efficiency of extraction also increased. Beyond 15 minutes it was more or less constant with slightly higher extraction recovery at 20 minutes. But with 20 minutes issue of repeatability occurred as drop tends to dislodge

in the aqueous sample while retracting the plunger of syringe. With this view, 15 minutes was optimized as the extraction time.

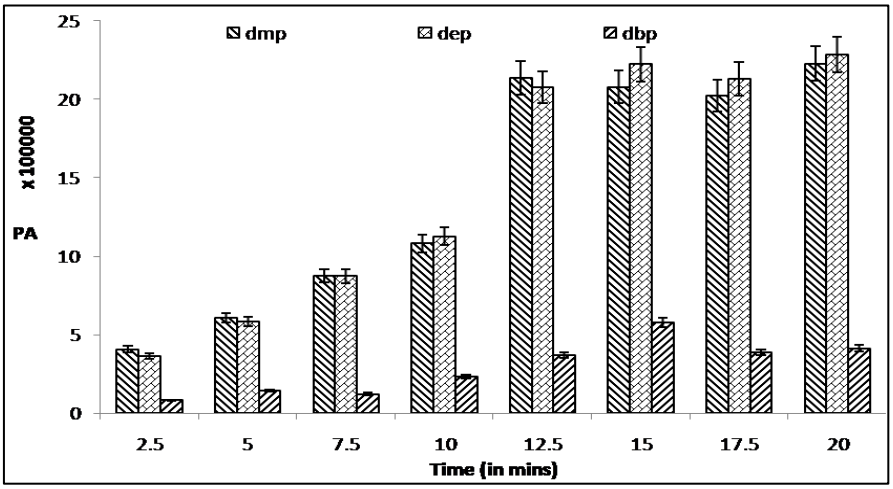


Figure 35 Effect of extraction time on extraction of analytes

Effect of depth of organic droplet:

Depth of organic droplet was the depth ***(figure 36)*** from the rim of aqueous sample in the till 0.8 cm, 1.0 cm and 1.2 cm ***(figure 37)***. 1.0 µg mL^{-1} spiked sample with target analytes was extracted using the parameters optimized so far. And at a depth of 1.2 cm higher extraction was achieved as it was exactly in the centre of sample giving it the uniform exposure for target analytes to get extracted and beyond this depth at a stirring rate of 300 rpm, the drop dislodged ***(table 27)***. So, a depth of 1.2 cm was optimized for further experiments.

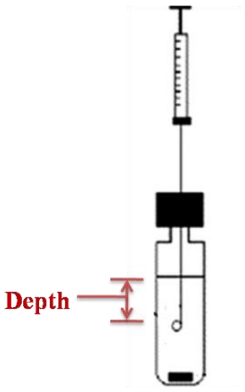


Figure 36 Depth of droplet

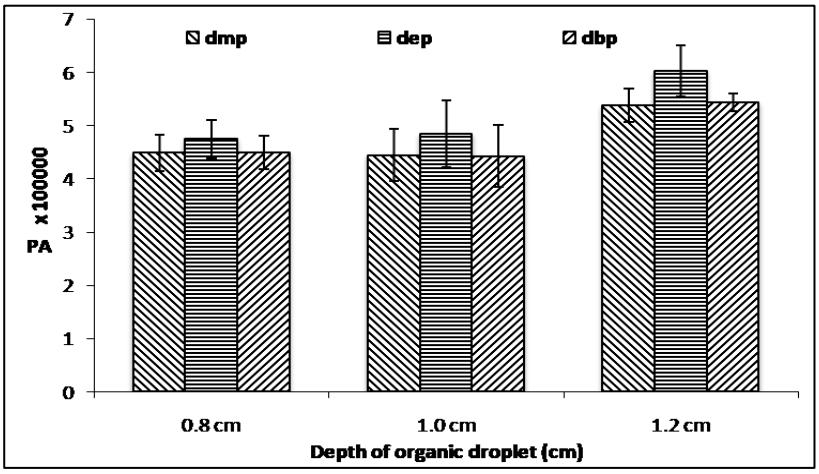


Figure 37 Effect of droplet volume in the sample

Dept of Organic drop (cm)	*DMP*	*DEP*	*DBP*
	Average peak area in GC (n=3)		
0.8	448705	474402	448652
1.0	444037	484111	442077
1.2	537797	602305	543494

Table 27 Effect of depth

Effect of volume of aqueous sample:

Volume of aqueous sample was varied from 5 mL to 15 mL by keeping the concentration (1.0µg mL^{-1}) of analytes constant, stirring rate 300 rpm, extraction time 15 minutes, depth 1.2 cm and other optimized parameters constant. Maximum extraction was achieved at 10 mL.

Vol of aqueous sample	*DMP*	*DEP*	*DBP*
	Average peak area in GC (n=3)		
5.0	341890	383729	367379
7.5	349300	358311	318005
10.0	467541	497912	457275
12.5	377605	400434	359796
15.0	367759	439813	454399

Table 28 Effect of aqueous sample

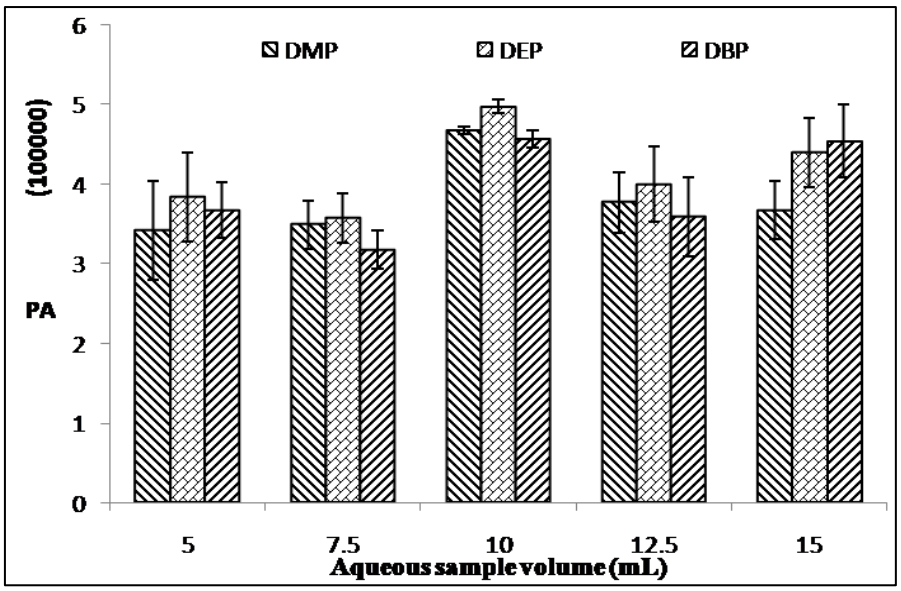


Figure 38 Effect of volume of aqueous sample

Effect of temperature:

To study the effect of temperature on extraction of the analytes, 1.0 µg mL^{-1} spiked sample was extracted by introducing 1.0 µL drop of toluene in the sample ***(figure 39)***.

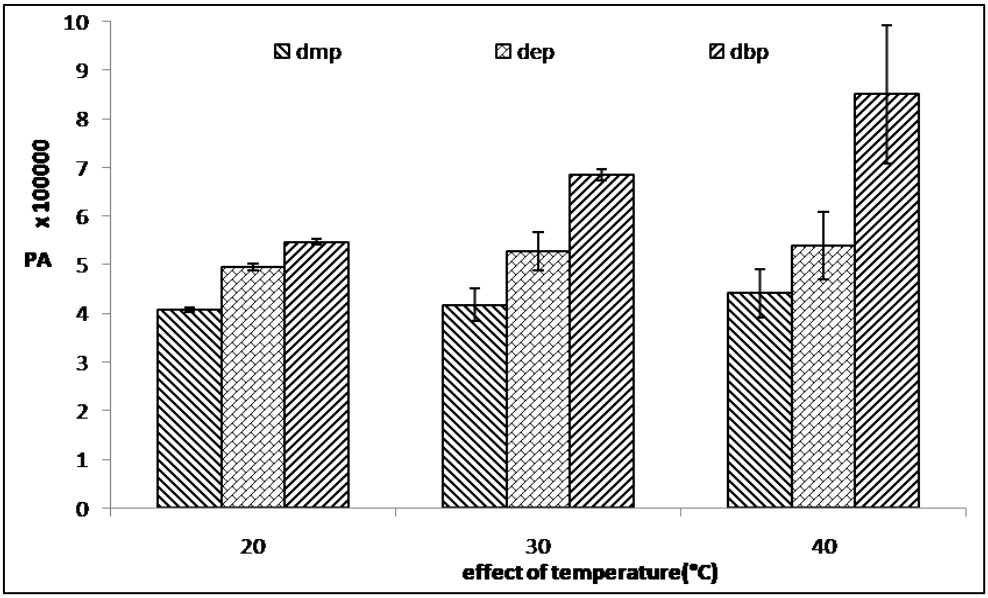


Figure 39 Effect of temperature

Temperature (°C)	*DMP*	*DEP*	*DBP*
	Average peak area in GC (n=3)		
20	407632	417712	441437
30	495601	527605	539837
40	546708	684723	850460

Table 29 Average peak area for the study of effect of temp

The analysis was performed in triplicate for each temperature (20 °C, 30 °C and 40 °C) study and the other parameters of stirring rate, extraction time and volume of organic droplet were kept constant as optimized. It was observed that as temperature increased ***(table 29),*** due to increase in the diffusion rate of analytes, their extraction in the solvent increased. Study at higher temperatures further was not performed due to effect on reproducibility of droplet (which dislodged at higher temperatures). The extraction efficiency for dimethyl phthalate, diethyl phthalate were in the same range but that for di-*n*-butyl phthalate was highest at 40 °C but the variation was also higher in that case. Thus, 30 °C was chosen as the optimized temperature.

Effect of salt addition:

By increasing the ionic strength (0 to 7.5 %, w/v) of an aqueous sample by the addition of NaCl salt, the extraction of the analytes increases. In a spiked sample of 1.0 µg mL^{-1} with analytes whose extraction was performed with the parameters as optimized. It was observed in the study that as the addition of salt increases extraction of the analytes decreases ***(figure 40)***. This was attributed to the reason that the salt added in the aqueous sample might have altered the physical properties (and behaviour) of the Nernst diffusion film, which is the interface for the analytes extraction, thereby decreasing their diffusion rate in the droplet [16].

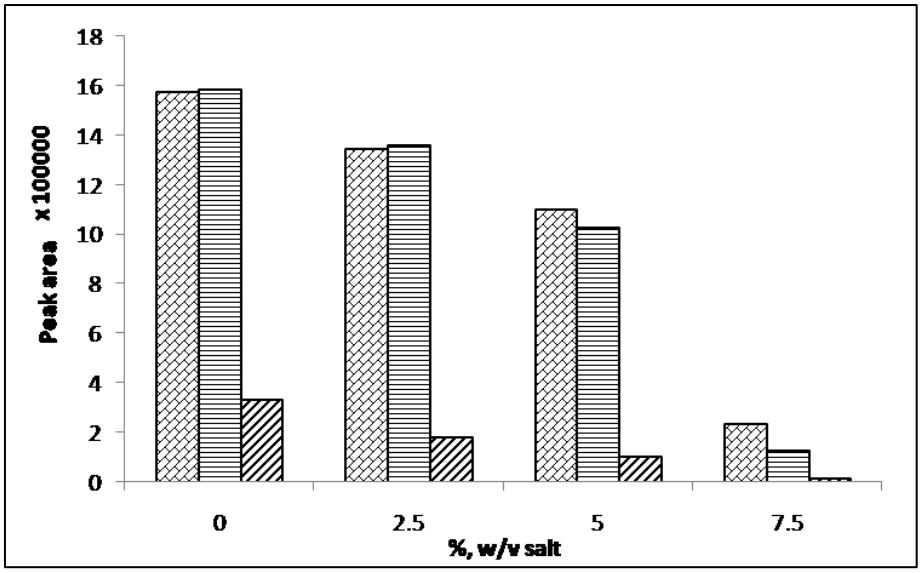


Figure 40 Effect of salt addition

This behaviour exhibited by the analytes on addition of salt was studied by Psillakis and Kelogerakis. They determined the concentration of nitroaromatic explosives in water sample by single drop microextraction [16 and 17]. Therefore, no salt was added in the experiments so as to achieve higher extractions of the analytes ***(table 30)***.

Salt addition (w/v) %	***DMP***	***DEP***	***DBP***
	Average peak area in GC (n=3)		
0	1575105	1583046	332785
2.5	1346015	1360841	181648
5.0	1099246	1028575	104278
7.5	233345	125490	15346

Table 30 Effect of salt addition

Effect of geometry of vessel on extraction:

Shape of container can affect rate of diffusion of molecules and hence extraction recovery of the analyte. To demonstrate this effect the following experiments were conducted. A 15 mL glass vial (1.6 cm depth and 4.0 cm wide), 25 mL capacity conical flask (4.4 cm depth and 4.0 cm wide) and 10 mL capacity volumetric flask (2.8 cm depth and 2.1 cm wide) were used in the study.10 mL aqueous sample spiked with analytes at a concentration of 1.0 µg mL^{-1} was extracted with 1.0 µL of toluene at 300 rpm for 15 minutes. It was observed that highest extraction was achieved in 15 mL capacity glass vial ***(figure 41)***.

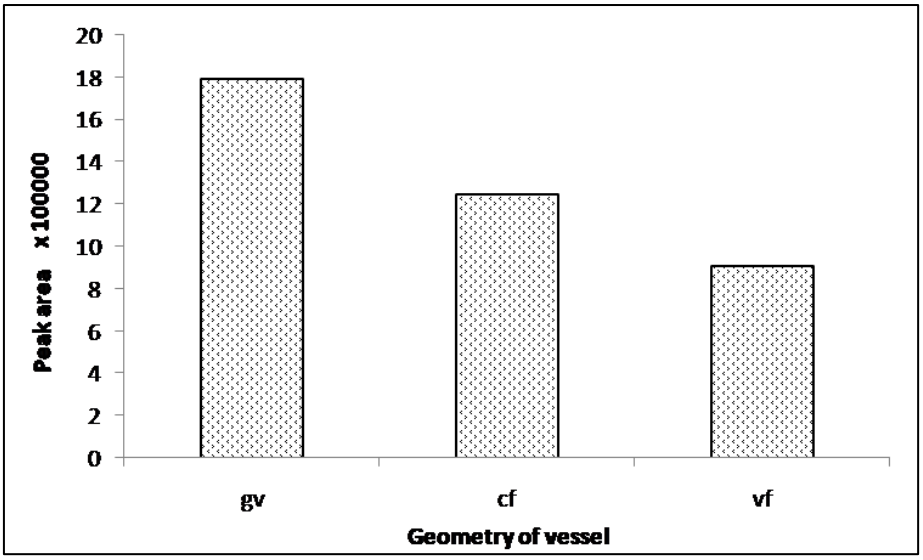


Figure 41 Effect of geometry of vessel

This would be because of the uniform geometry of the vessel throughout (which is not the case in volumetric flask and conical flask) and the optimum depth at which the droplet gets exposure with a safe distance from the magnetic stirring needle that doesn't allow the drop to dislodge from the tip. Therefore, 15 mL capacity glass vial was used in all the experiments.

Linearity and repeatability:

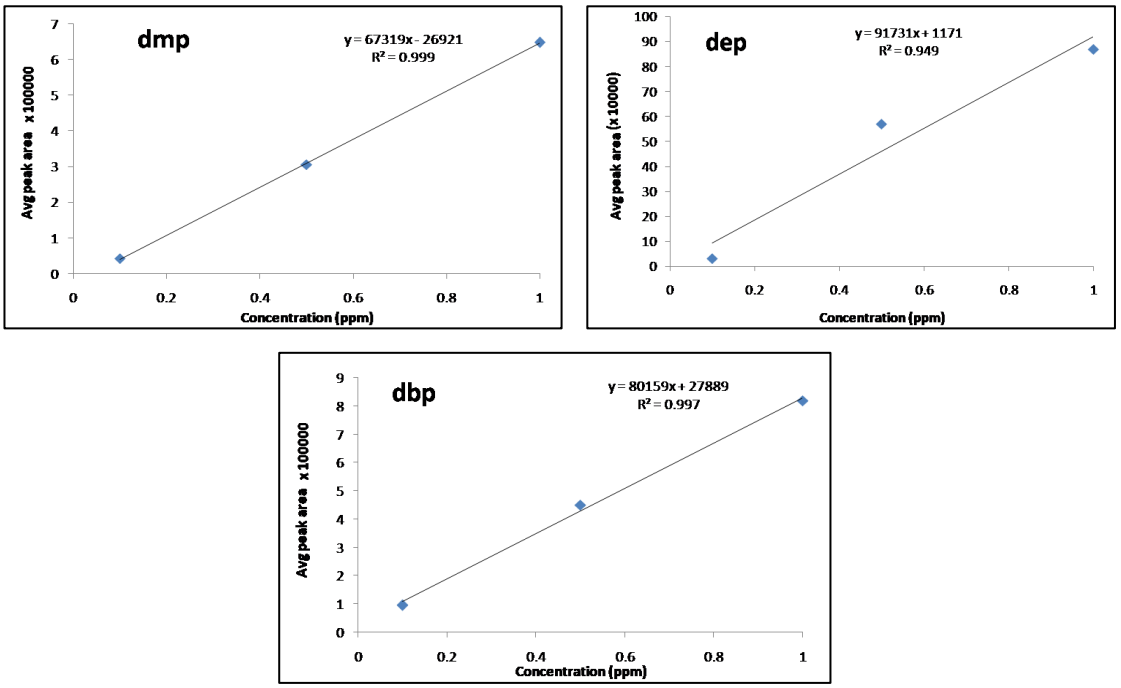


Figure 42 Graph for DMP, DEP and DBP plotted after extraction

Linearity was observed ***(figure 42)*** in the extraction carried out with the optimized parameters for the three target analytes spiked at a concentration of 1.0 µg mL^{-1}, 0.5 µg mL^{-1} and 0.1 µg mL^{-1}. Repeatability of the technique was determined by repeating five replicate injections of spiked sample with the target analytes extracted from 1.0 µg mL^{-1} 10 mL aqueous sample with optimized parameters and using toluene as extraction solvent. The coefficient of variation (%RSD) was calculated from the peak responses obtained after extraction for each target analyte in table 31 for DMP, table 32 for DEP and table 33 for DBP.

Target analyte	***Peak response***	***Average***	***Std deviation***	***% RSD***
DMP	648085	646703.2	23148.0	3.57
	621268			
	650428			
	631629			
	682106			

Table 31 Precision (% RSD) for DMP

Target analyte	*Peak response*	*Average*	*Std deviation*	*% RSD*
DEP	869518	861636.2	37341.8	4.33
	892442			
	833884			
	812945			
	899392			

Table 32 Precision (%RSD) for DEP

Target analyte	*Peak response*	*Average*	*Std deviation*	*% RSD*
DBP	825821	843110.6	30361.0	3.60
	820086			
	886812			
	863126			
	819708			

Table 33 Precision (%RSD) for DBP

In all the cases the %RSD was within 5, considered to be a reasonably good range.

Comparison of the extraction techniques:

The purpose of our study was to compare the three microextraction techniques and it was achieved by performing comparison of these techniques on the basis of the statistical data generated.

After the optimization of the parameters for all the three techniques, they were compared as follows ***(table 34)***:

Parameters	**SPE**	**DLLME**	**SDME**
LDR	0.5 to 10 µg mL^{-1}	1.0 to 100 µg mL^{-1}	1 to 80 µg mL^{-1}
R^2	0.9986 to 0.9994	0.9995 to 0.9969	0.9932 to 0.9989
LOD	0.08 µg mL^{-1}	0.07 µg mL^{-1}	0.04 µg mL^{-1}
LOQ	0.27 µg mL^{-1}	0.23 µg mL^{-1}	0.14 µg mL^{-1}
Precision (%RSD)	4.26 %	3.15 %	4.39 %
ER(%) or EF	25 to 98	90 to 96	92 to 152

Table 34 Comparison of the three microextraction technique

Based on the above studies real water samples were analysed by single drop microextraction technique as lower detection limits were achieved.

Sample	DMP	DEP	DBP
Tap water from lab No.4	ND	ND	D (1.02µg mL^{-1})
Aquafina bottled water	ND	ND	ND
Sama lake water sample	ND	ND	D(2.99 µg mL^{-1})
Harni lake water sample	D (0.86 µg mL^{-1})	ND	ND

Table 35 Determination of phthalate esters in real samples by SDME (n = 3)

The samples which showed presence of target analytes were subjected to GC-MS analysis and it was confirmed by the mass spectrum that the peak obtained in mass spectrum belonged to dimethyl phthalate as determined by GC in case of 500 mL Harni lake water sample, S1 (Lat: 22°20'17", Long: 73°13'11") (figure 43).

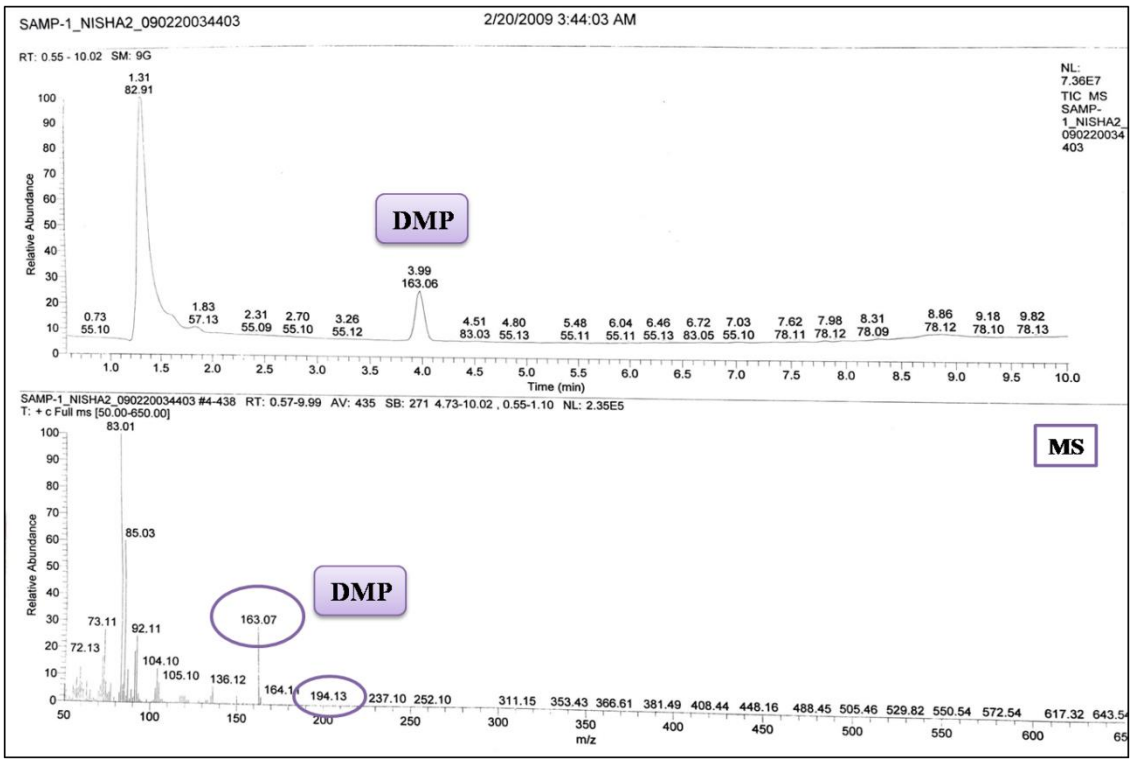


Figure 43 GC-MS spectrum for dimethyl phthalate determined in Harni water sample

Based on the study performed so far we have developed a ternary component set up for the extraction of target analytes from another real sample – ***oil*** (250 mL ground nut oil packed in polyethylene bag procured locally from Sayajipura village grocery store). Plastic packaging of vegetable oils can be a source for the migration of phthalates into the oils as during transportation they tend to undergo harsh conditions (high temperature conditions, not proper storage conditions with controlled temperature conditions), rough handling, etc. As phthalates are fat soluble, they can

leach out and migrate into the oil. In oil, directly the extraction solvent cannot be introduced as the droplet formation doesn't occur due to difference in density, surface tension as the syringe passes through the oil layer, the surface of syringe gets coated with oil, thus inhibiting the formation of droplet. Therefore, to overcome this situation, we have used single drop microextraction, wherein the droplet containing the extraction solvent is introduced but with a modification. The experimental set up for the extraction is shown in figure 44. Initially, 10 mL milli-Q water was introduced in the glass vial and then 2 mL oil was introduced in the glass vial and then in the end the extraction solvent was introduced through the syringe but as oil floats on water. The oil came in contact with the syringe, the moment it passed through oil and then reached the water layer, the plunger of the syringe was pressed in order to form the droplet but as the syringe then had the coating of oil layer, the droplet formed and dislodged the moment it was introduced.

To overcome this, we first introduced milli-Q water in the glass vial and then introduced the syringe containing the extraction solvent. The droplet was not pressed through the plunger of micro syringe till we had not introduced the oil layer. In short, the oil layer was introduced at last. Since, the extraction was not occurring directly from oil but via water, it would have resulted in no extraction within 15 minutes. Also there, the equilibrium concentration of analytes between oil and water needed to take place first. For this without the drop formation, this oil-water mixture was stirred at 300 rpm for 5 minutes so as to establish diffusion of analytes from oil into water which were then extracted in the extraction solvent droplet introduced at the end. Further the extraction was carried out for 15 minutes.

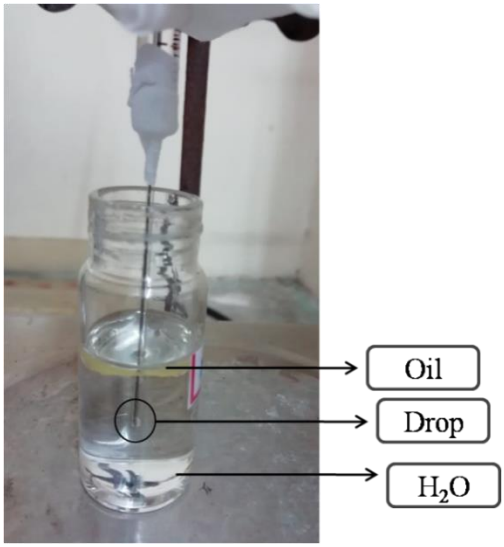


Figure 44 SDME in oil sample

Analyte	*Extraction recovery (ER)in oil sample*		*ER (%)*
	Blank	Spiked (5 µg mL^{-1})	
DMP	ND	1.29 µg mL^{-1}	25.8
DEP	ND	2.38 µg mL^{-1}	47.8
DBP	D (0.68µg mL^{-1})	1.43 µg mL^{-1}	28.6

Table 36 Extraction recovery of phthalates in oil by SDME

Table 36 exhibits the extraction recovery of phthalates in blank and spiked oil sample. The extraction recoveries were lower because the analytes could not be determined directly in oil samples. But still the method showed ability for the detection of analytes in oil samples via a three phase system.

Conclusion

Thus, with the optimization of parameters for each technique and their comparison, we were able to achieve higher enrichment factors for all the analytes with single drop microextraction (SDME). And therefore, we applied this technique for the determination of target analytes in real water samples and oil sample. Comparison of the technique based on our observations during the experiments performed.

Technique	*Pros*	*Cons*
SPE	Easy experimental set up.	Cost of adsorbent, drying after loading step was critical.
DLLME	Cost effective, dispersion of small drops during injection that leads to higher extraction performance	Getting sample from the sedimented phase can be critical, if the centrifuge tube does not have conical bottom.
SDME	Easy experimental set up, cost effective, higher enrichment factors	Handling and stability of drop.

References

1. J He, R Lv, J Zhu, K Lu. Selective solid-phase extraction of dibutyl phthalate from soybean milk using molecular imprinted polymers. *Analytica Chimica Acta* **2010**, 661, 215–221. doi: 10.1016/j.aca.2009.12.029

2. H Farahani, P Norouzi, R Dinarvand, M R Ganjali. Development of dispersive liquid–liquid microextraction combined with gas chromatography–mass spectrometry as a simple, rapid and highly sensitive method for the determination of phthalate esters in water samples. *Journal of Chromatography A* **2007**, 1172, 105–112. doi: 10.1016/j.chroma.2007.10.001.

3. M D Carlo, A Pepe, G Sacchetti, D Compagnone, D Mastrocola, A Cichelli. Determination of phthalate esters in wine using solid-phase extraction and gas chromatography–mass spectrometry. *Food Chemistry* **2008**, 111, 771–777. doi: 10.1016/j.foodchem.2008.04.065.

4. J I Cacho, N Campillo, P Vinas, M H Córdoba. Determination of alkylphenols and phthalate esters in vegetables and migration studies from their packages by means of stir bar sorptive extraction coupled to gas chromatography–mass spectrometry. *Journal of Chromatography A* **2012**, 1241, 21– 27. doi: 10.1016/j.chroma.2012.04.018.

5. K S Kumar, P B Samnani. Preconcnetration and qualitative determination of esomeprazole magnesium present in water. *Analytical Letters* **2010**, 43, 1427 – 1433. doi: 10.1080/00032710903502058

6. ICH Harmionised tripartite guidelines. Validation of analytical procedures: text and methodology Q2 (R1). *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use* **2005.**

7. Shrivastava and Gupta. Methods for LOD and LOQ determination. **2011**, 2, 11.

8. An analyst's guide to precision. *Analytical Methods* **2015**, 7, 8508. doi: 10.1039/c5ay90071a

9. J M Betz, P N Brown, M C Roman. Accuracy, precision, and reliability of chemical measurements in natural products research. *Fitoterapia* **2011**, 82, 44 – 52. doi: 10.1016/j.fitote.2010.09.011.

10. Y Assadi, M R M Hosseini, E Aghaee, F Ahmadi, S Berijani. Determination of organic compounds in water using dispersive liquid–liquid microextraction.

Journal of Chromatography A **2006**, 1116, 1–9.doi: 10.1016/j.chroma.2006.03.007.

11. L Xue, D Zhang, T Wang, X wang and X. Du. Dispersive liquid-liquid microextraction followed by high performance liquid chromatography for determination of phthalic esters in environmental water samples. *Analytical Methods* **2013**, doi: 10.1039/C3AY41996G.

12. M A Farajzadeh, S R Aghdam, M R A Mogaddama and H Ghorbanpour. An elevated temperature-dispersive liquid–liquid microextraction method combined with GC-flame ionization detection as a sensitive method for determining phthalate esters. *Analytical Methods* **2015**. doi: 10.1039/c4ay02998d.

13. L Xu, C Basheer, H K Lee. Developments in single-drop microextraction. *Journal of Chromatography A***2007**, 1152, 184–192. doi: 10.1016/j.chroma.2006.10.073

14. Q Xiao, B Hu, C Yu, L Xia, Z Jiang. Optimization of a single-drop microextraction procedure for the determination of organophosphorus pesticides in water and fruit juice with gas chromatography-flame photometric detection. *Talanta* 2006, 69,848–855. doi: 10.1016/j.talanta.2005.11.024.

15. M Saraji, M Bakhshi. Determination of phenols in water samples by single drop-microextraction followed by in-syringe derivatization and gas chromatography-mass spectrometric detection. Doi: 10.1016/j.chroma.2005.08.063.

16. M C Lopez-Blanco, S. Blanco-Cid, B. Cancho-Grande and J. Simal-Ga´ndara. Application of single-drop microextraction and comparison with solid-phase microextraction for the determination of α- and β-endosulfan in water samples by gas chromatography-electron capture detection. *Journal of Chromatography A***2003**. 984, 245–252.

17. E Psillakis and N Kelogerakis. Application of solvent microextraction to the analysis of nitroaromatic explosives in water. *Journal of Chromatography A* **2001**. 907, 211.

•