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Determination of Endosulfan in Water Samples Using Dispersive Liquid-liquid Micro-extraction and Experimental Design for Optimization

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ABSTRACT: Water contamination due to the wide variety of pesticides used in agriculture is a global environmental pollution problem. In order to reach at sub-μgL^{-1} levels of detection, an efficient extraction technique is required. A simple, fast and economical method, dispersive liquid-liquid micro extraction (DLLME), followed by gas chromatography-mass spectrometry was assessed for determining endosulfan in water samples. Experimental parameters which control the performance of DLLME, such as extraction and disperser solvents type and their volumes, temperature, and salt addition were studied by experimental design. The main factors affecting the extraction efficiency, volumes of disperser and extraction solvents, were optimized by response surface method. Under optimum conditions, the method was linear over the range 0.1-50 µg/L. The enrichment factor and extraction recovery were 163.4 and 63.73, respectively. Correlation coefficient and limit of detection (LODs) are 0.9996, 20 ng/L, respectively.

Key words: Endosulfan, Pesticide, Dispersive liquid-liquid micro extraction, Gas chromatography-mass spectrometry, Experimental design

INTRODUCTION

Endosulfan is a chlorinated hydrocarbon pesticide of the cyclodiene subgroup which acts as a contact poison in a wide variety of insects and mites. It can also be used as a wood preservative. Endosulfan (a mixture of two stereoisomer, α- and β- endosulfan), as other organochlorine pesticides, persists in the environmental media and has the ability of bioaccumulation and biomagnifying in food chains (Cabaleiro *et al.,* 2008; Dutta and Dalal, 2008). Exposure to endosulfan can occur through inhalation, ingestion, eye or skin contact. It causes central nervous system and respiratory effects in humans. The greatest potential for adverse effects of pesticides is through contamination of the hydrologic systems (El Bakouri *et al.,* 2005).Endosulfan does not easily dissolve in water. It does stick to soil particles readily. Transport of this pesticide is most likely to occur if endosulfan is attached to soil particles in surface runoff. It has, however, been detected in well and surface waters near areas of application at very low concentrations, and also in drinking waters due to the fact that some of these waters are used for drinking (El Bakouri *et al.,* 2005, Schäfer *et al.*,2008).Monitoring pesticide residues in waters is important for human health protection and environmental control.Endosulfan can be extracted from aqueous matrices using a variety of conventional techniques including liquid–liquid extraction (LLE) [Brito et al. 2002, Columé et al. 2000, Sankararamakrishnan *et al.,* 2005) and solid-phase extraction (SPE) El Bakouri *et al.,* 2008). LLE technique is time consuming, expensive and hazardous to health due to the high volume of toxic solvents used. SPE needs less solvent, but is still time consuming, and often requires a concentration stage that presents disadvantages such as losses in the evaporation step, risks of contamination, and loss of sensitivity due to the injection of only a small aliquot of the sample (Basheer *et al.,* 2002).

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Solid-phase micro extraction (SPME) (Li *et al.*, 2003, Aguilar *et al.*, 1998) and liquid-phase micro extraction (LPME) using single drop solvent (López-Blanco *et al.*, 2003, are more recent extraction procedures. For SPME, limited fiber life, fiber breakage, stationary-phase bleeding, competitive absorption, and the relatively high cost of fibers have been reported by users of SPME. Some disadvantages of LPME are fast stirring which may cause break up the organic solvent drop and air bubble formation; it is time-consuming and in most cases equilibrium is not attained even after a long time (Ahmadi *et al.*, 2006).

Recently, a simple and rapid pre-concentration and micro extraction method, dispersive liquid–liquid micro extraction (DLLME) is developed by some researchers (Rezaee *et al.*, 2006, Berijani *et al.*, 2006, Farajzadeh *et al.*, 2007, Shokoufi *et al.*, 2007, García- López *et al.*, 2007, Fariña *et al.*, 2007). Being independent of time is the most important advantage of this method. Rapidity, high enrichment factor, low cost, simplicity and ease of use, requiring no conditioning (as is the case with the fiber in the solid-phase micro extraction) and no need for instrument modification are some of the advantages of this method (Rahnama Kozani *et al.*, 2007).In this study, our objective was to develop, optimize and validate a simple and efficient extraction method, DLLME, combined with gas chromatography-mass spectrometry for determination of endosulfan in water samples. The optimization of the method was performed using experimental design to obtain the optimum conditions.

MATERIALS & METHODS

Analytical standard grade of Endosulfan was purchased from Riedel-de Haën (Hannover, Germany). Other chemicals including chloro-benzene, chloroform, carbon tetrachloride, ethanol, methanol, acetone and sodium chloride with purity higher than 99% were supplied by Merck chemical company (Merck, Darmstadt, Germany). Stock standard solutions (1000 mg/L) were prepared in methanol. Intermediate standard solutions were prepared by diluting the stock standard solutions in methanol. Water samples were prepared by spiking different volumes of intermediate standard solutions in bid stilled water. All solutions were stored at 4°C in dark. Surface, well and tap water samples, used for evaluation of the method were collected from Tehran (Iran). GC-MS analyses were performed on a HP-6890 GC system coupled with a 5973 network mass selective detector and equipped with a HP5-MS capillary fused silica column (60 m; 0.25 mm I.D.; 0.25 µm film thickness; methyl 5% phenyl polysiloxane). The oven temperature program initiated at 100 ºC, held for 1 min then ramped at 30 °Cmin^{-1} to 250 °C held for 3 min. A split/splitless injector was used in the splitless mode (1 min) for DLLME analyses. Other operating conditions were as follows: carrier gas, He (99.999%); with a flow rate of 1 mL/min; injector temperature, 220 ºC. Mass spectra were taken at 70 eV. Mass range was from mz^{-1} 20–500 emu. Injection into GC-MS was carried out using a 1µL micro syringe model Hamilton 7001. For investigation of temperature effect, julabo U3 water bath (Seelbach, Germany) were used. Centrifuges were performed by Hermle Z 200 A centrifuge instrument (Wehingen, Germany).

Dispersive liquid-liquid micro extraction procedure consists of two steps: (1) the injection of an appropriate mixture of extraction and disperser solvent into aqueous sample containing analytes resulting in the formation of a cloudy solution. (2) The centrifugation of cloudy solution. After centrifugation, the determination of analytes in sediment phase can be performed by instrumental analysis. Because of infinitely large surface area between extraction solvent and aqueous sample, the equilibrium state is achieved quickly and extraction is independent of time. So, under optimum conditions, 2 mL of each sample was placed in a 10 mL screw cap glass tube with conic bottom, and 0.5 mL of methanol (as disperser solvent) containing 40 µL chloroform (as extraction solvent) was injected rapidly into each sample solution using a 1.00 mL syringe. The mixture was centrifuged for 3 min at 4500 rpm using the centrifuge. The dispersed fine particles of extraction solvent separated and settled at the bottom of conical tube. 0.5 µL of the separated phase was removed using a 1.0 µL micro syringe and injected into the GC-MS. Finally, the statistical software package, Design-Expert 7.1.3, was used for analysis of the experimental data and also to plot the response surface graphs.

RESULTS & DISCUSSION

It is necessary to choose a suitable organic extraction solvent. It should have higher density rather than water, good affinity for target compounds, low solubility in water so as to prevent the dissolution in the aqueous phase and excellent gas chromatographic behavior. On the basis of these considerations, chloro-benzene (density: 1.11 g/mL), carbon tetrachloride (density: 1.59 g /mL), and chloroform (density: 1.47 g/mL) were tested in the preliminary experiments. The main point for selection of disperser solvent is its miscibility in the organic phase (extraction solvent) and aqueous sample solution. Acetone, ethanol and methanol were assayed for this purpose. Fig. 1 compares the peak area as the extraction efficiency for different extraction and disperser solvents. It could be seen that chloroform as extraction solvent with methanol as disperser solvent gave the maximum efficiency.

The traditional optimization procedure varying "one variable at a time", is a strategy "based on experience, educated guesswork and luck" that does not guarantee the attainment of a true optimum of the extraction conditions. Conversely, the chemo-metric approach relies on a rational experimental design, which allows the simultaneous variation of all experimental factors, saving time and materials (Gonçalves *et al.*, 2006). The full factorial design was chosen to determine the most significant factors. Although a factorial design does not generate the information required for complete modeling of the response surface, it could extract significant information with a minimum number of test runs. With a factorial design it is possible to determine the main (or linear) effects as well the interactive effects of the selected factors (Kim *et al.*, 2002). Two levels full factorial design requires an experiment to be carried out at all possible combinations of the two levels of each factor considered (Massumi *et al.*, 2002).The following factors were evaluated: extraction and disperser solvent volumes, temperature and ionic strength of the sample. Therefore, a full factorial design included $2^4 = 16$ experiments developed. The experiments were run in a random manner in order to minimize the effect of uncontrolled variables. Because the run time was not enough to perform all the 16 experiments during one working day, they were divided into two blocks, each with eight experiments. The peak area (sum of α- and β-endosulfan) was considered as the experimental response.Table 1 lists the factors, the corresponding symbols and levels; and Table 2 shows the experimental design matrix and the results derived from each run.

Table 1. Factors and their levels in full factorial design

Factor	Symbol	Levels	
		-1	+1
Volume of extraction solvent (µL)	E	40	60
Volume of disperser solvent (mL)	D	0.2	0.5
Temperature (C)	T	20	50
Salt amount (g)	S	0	0.1

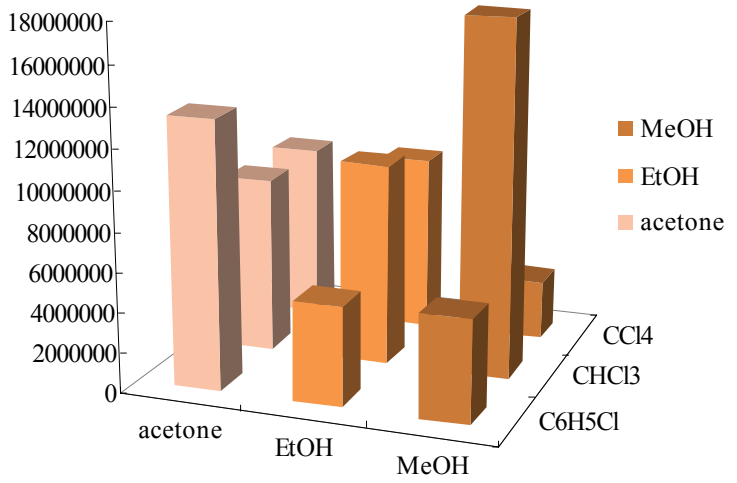

Fig. 1. Selection of extraction and disperser solvents using GC-MS. Extraction conditions: sample volume, 2 mL; volume of disperser solvent, 0.4 mL; volume of extraction solvent, 30 µL

Table 2. Design matrix and responses for full factorial design

Block	Run No.	E	D	T	S	Response	Effect
1	1	+1	+1	+1	-1	8155484	EDT
1	2	+1	+1	-1	+1	6615440	EDS
1	3	-1	-1	-1	+1	8443095	S
1	4	-1	+1	-1	-1	12415831	D
1	5	-1	-1	+1	-1	10412995	T
1	6	+1	-1	+1	+1	5506920	ETS
1	7	-1	+1	+1	+1	18079503	DTS
1	8	+1	-1	-1	-1	6877994	E
2	9	-1	-1	-1	-1	11044450	Average
2	10	-1	+1	+1	-1	29626418	DT
2	11	+1	+1	+1	+1	6301372	EDTS
2	12	-1	-1	+1	+1	10312887	TS
2	13	+1	-1	+1	-1	5193404	ET
2	14	+1	+1	-1	-1	6819438	ED
2	15	+1	-1	-1	+1	5285518	ES
2	16	-1	+1	-1	+1	9866965	DS

The significance of the effects was checked by analysis of the variance (ANOVA) and *F*-value significance level using software package, Design-Expert 7.1.3. Generally, the statistical significance of effects in an ANOVA table can be estimated by the *p*-value generated from the hypothesis test. If the *p*-value of any effect is lower than 0.0500 (95% confidence), the effect is considered to be statistically significant. As shown in Table 3. E and D are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. In addition, the ANOVA table shows that there is no significant effect due to the blocking. The same results were obtained by doing the probability normal plot. Fig. 2. the normal probability plot of standardized effects, shows graphically the effect of each factor and interactions. Each point on the plot represents an effect; the effects that are not statistically significant are located close to the reference line and are left unlabeled. The effects represented by points far from the reference line are considered statistically significant (Kim *et al.*, 2002). The temperature and ionic strength had no significant effect on the response. Hence, the two factors of E and D were to be used in the next step of the design.

Central composite design (CCD) can be used for systematic optimization and it offers an efficient route for rapid optimization of resolution with multiple interacting factors. The CCD is build up of a full factorial 2^f design to which a star design is added. The CCD is completed by addition of a center point (Jančić *et al.*, 2008). In this step, a rotatable, orthogonal CCD was employed to determine the optimum conditions for the critical factors. This design permitted the response surface to be modeled by fitting a second-order polynomial with the number of experiments equal to $(2^f+2f + n)$, where f is the number of factors and n is the number of center runs (Mousavi *et al.*, 2007). From the repetition of the center point, the experimental variance at the center of the domain can be estimated (Jančić *et al.*, 2008). Using Eq. (1) the axial spacing of $a = \pm 1.414$ was calculated to satisfy rotate-ability. Then, N_0 was obtained using Eq. (2) equal to 8

$$a = \sqrt[4]{N_f} \qquad (1)$$

$$a = \sqrt{\frac{\sqrt{(N_f + N_a + N_o)N_f} - N_f}{2}} \qquad (2)$$

Where N_f is the number of factorial points (2^f), N_a is the number of extra star points, ($2f$), and N_0 is the number of runs at the center of design. The factor levels used in the CCD and the corresponding design matrix and responses are shown in Tables 4 and 5, respectively (Mousavi *et al.*, 2007). In the final step of the design, a

Table 3. Analysis of variance table (ANOVA) for full factorial design.

Source	Sum of squares	df [a]	Mean square	F value [b]	p-value prob >F [c]	significance
Block	2.147×10^{-16}	1	2.147×10^{-16}	1.02	0.3697	not significant
Model	3.202×10^{-14}	10	3.202×10^{-15}	15.20	0.0092	significant
E	2.363×10^{-14}	1	2.363×10^{-14}	112.20	0.0004	
D	4.246×10^{-15}	1	4.246×10^{-15}	20.16	0.0109	
T	4.595×10^{-16}	1	4.595×10^{-16}	2.18	0.2137	
S	1.307×10^{-15}	1	1.307×10^{-15}	6.21	0.0674	
ED	1.246×10^{-19}	1	1.246×10^{-19}	5.917×10^{-4}	0.9818	
ET	1.087×10^{-15}	1	1.087×10^{-15}	5.16	0.0856	
ES	3.166×10^{-19}	1	3.166×10^{-19}	1.503×10^{-3}	0.9709	
DT	1.104×10^{-15}	1	1.104×10^{-15}	5.24	0.0839	
DS	2.834×10^{-17}	1	2.834×10^{-17}	0.13	0.7324	
TS	1.527×10^{-16}	1	1.527×10^{-16}	0.72	0.4425	
Residual	8.425×10^{-16}	4	2.106×10^{-16}			
Cor total [d]	3.307×10^{-14}	15				

[a] Degrees of freedom
[b] Test for comparing model variance with residual (error) variance
[c] The probability value associated with the F Value
[d] Sum of squares total corrected for the mean

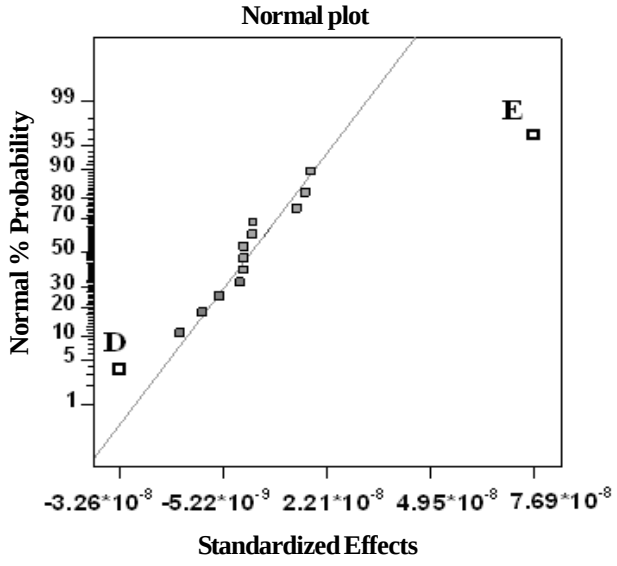

Fig. 2. Normal probability plot for full factorial design 2^4 to find important factors

response surface model was developed by considering all the responses in the CCD using the software package, Design-Expert 7.1.3. In developing the final model, main effects, two and higher order interaction effects and curvatures were applied in coded forms. Then, the model with the most reasonable statistics, that is, higher *F*- and *R*-values and low standard error was considered as the satisfactory response surface model. The model consisted of two main effects, one two-factor interaction effect and two curvature effects. This model and its related statistics in terms of coded factors are shown in Eq. (3):

$$\text{Response} = b_0 + b_1E + b_2D + b_3ED + b_4E^2 + b_5D^2 \quad (3)$$

$b_0=1.133\times10^7$;
$b_1=-3.994\times10^6$;
$b_2=1.335\times10^6$;
$b_3=-1.487\times10^6$;
$b_4=5.987\times10^5$;
$b_5=-2.336\times10^5$

In Eq. (3), the coefficient for E (b_1) is large and negative. This means that the efficiency increases with decreasing this variable. The ED appears with a negative coefficient (b_3) which indicates E and D have opposite effects on theresponse.The b_2 value is less than the absolute value of b_1; therefore, it seems that the highly negative value of the b_1 more impresses the resultant rather than b_2. Fig.3 shows this interaction. The ANOVA data to

Table 4. Factor levels used in the central composite design

Factor	Symbol	Levels				
		-a	-1	0	+1	+a
Volume of extraction solvent (μL)	E	36	40	50	60	64
Volume of disperser solvent (mL)	D	0.14	0.20	0.35	0.50	0.56

Table 5. Design matrix and responses for the central composite design

Run	Block	E	D	Response
1	1	+1	-1	6352133
2	1	0	0	12059414
3	1	+1	+1	8240169
4	1	-1	-1	11465637
5	1	-1	+1	19301638
6	1	0	0	12854148
7	1	0	0	10993359
8	1	0	0	10875504
9	2	0	0	11038368
10	2	+1.414	0	7336855
11	2	0	0	11975761
12	2	0	-1.414	10871404
13	2	0	0	11131756
14	2	0	+1.414	11548752
15	2	-1.414	0	18379509
16	2	0	0	9665196

evaluate the significance of the model equation and model terms are shown in Table 6. The model F-value of 16.060 implies the model is significant. There is only a 0.03% chance that a model F-Value this large could occur due to noise. The "Lack of Fit (LOF) F-value" of 4.355 implies there is a 5.95% chance that a LOF this large could occur due to noise.

The analysis of the response surfaces can be done in several ways. The most immediate way of concluding the optimum conditions is the graphical inspection of the surfaces, since the 3D pictures give the complete overview of the systems (Armenta *et al.*, 2006). The main conclusion summarized by a 3D response surface plot (Fig. 4). It was observed that by decreasing the volume of the extraction solvent, sediment phase volume decreased, therefore, enrichment factor and response increased. An increase in response was obtained by increasing the methanol volume due to producing better cloudy solution and decreasing the sediment phase volume. As can be seen from Fig. 4. optimum condition is attained at high level of disperser solvent volume and low level of extraction solvent volume. The optimum and experimental responses are shown in Table 7. To evaluate the accuracy of the results obtained by the response surface model, three experiments were carried out under optimum conditions. As can be seen in Table 7. there is a good agreement

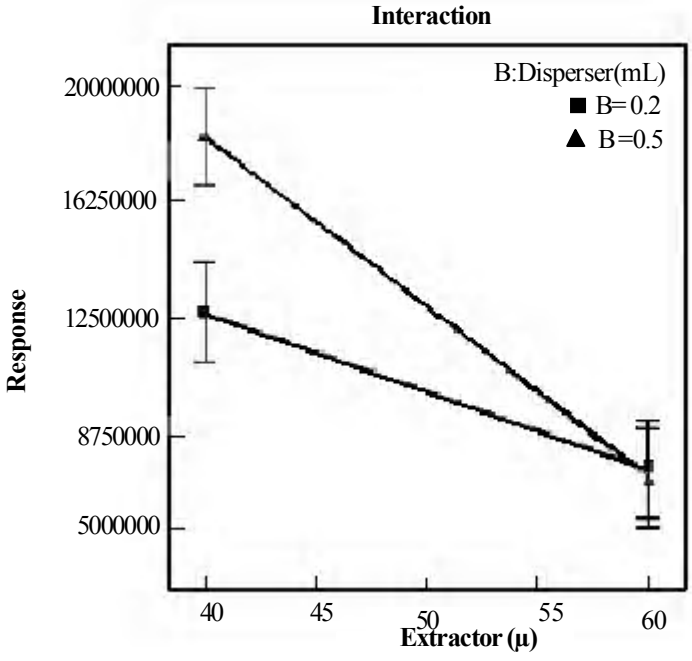

Fig. 3. Two-factor interaction of factors and their effects on the response

Table 6. Analysis of variance table (ANOVA) for response surface quadratic model

Source	Sum of squares	df [a]	Mean square	F value [b]	p-valueprob > F [c]	significance
Block	2.362×10^{9}	1	2.362×10^{9}	0.001	0.9755	not significant
Model	1.527×10^{14}	5	3.053×10^{13}	16.060	0.0003	significant
E	1.264×10^{14}	1	1.264×10^{14}	66.471	<0.0001	
D	1.426×10^{13}	1	1.426×10^{13}	7.501	0.0229	
ED	8.845×10^{12}	1	8.845×10^{12}	4.653	0.0594	
E^2	2.783×10^{12}	1	2.783×10^{12}	1.464	0.2571	
D^2	4.366×10^{11}	1	4.366×10^{11}	0.230	0.6432	
Residual	1.711×10^{13}	9	1.901×10^{12}			
Lack of fit [d]	1.173×10^{13}	3	3.908×10^{12}	4.355	0.0595	not significant
Pure error	5.384×10^{12}	6	8.973×10^{11}			
Cor total	1.698×10^{14}	15				

[a] Degrees of freedom
[b] Test for comparing model variance with residual (error) variance
[c] The probability value associated with the F Value
[d] The portion of the residual SS due to the model not fitting the data

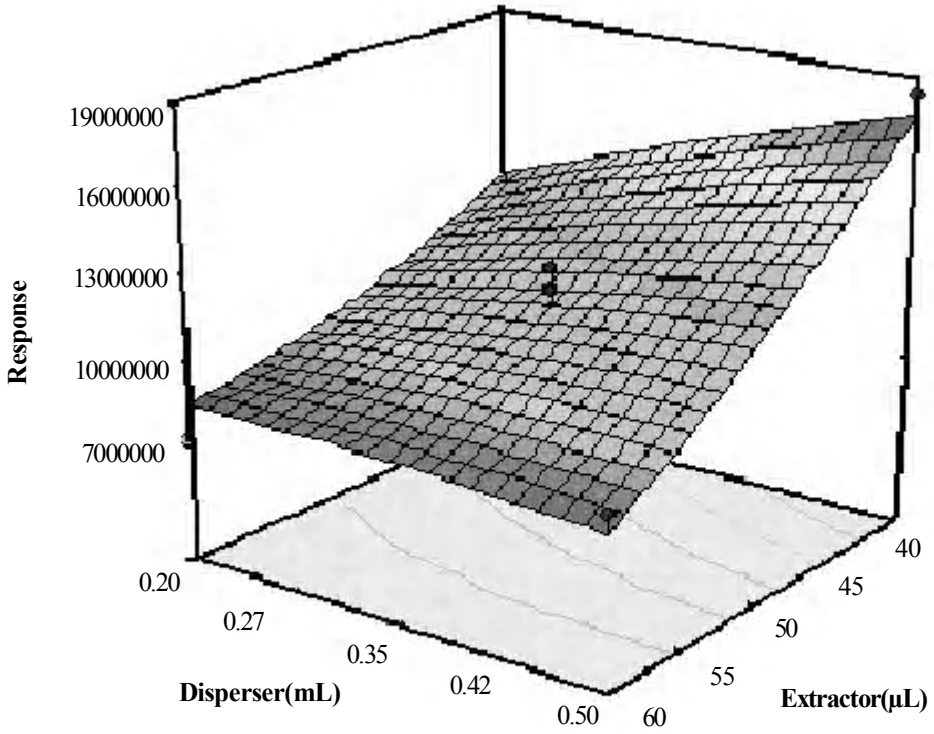

Fig. 4. Response surface for endosulfan extraction

Table 7. Optimum response and the corresponding levels

E (µL)	D (mL)	Optimum response	Experimental response [a]	% RSD [b]
40	0.5	1.851×10^{7}	1.808×10^{7}	5.6

[a] Mean value of three measurements.
[b] Relative standard deviation of three measurements.

between the calculated and experimental responses.Under the optimum condition, some analytical characteristics of the proposed DLLME method were obtained using GC-MS. The correlation coefficient (r^2), dynamic linear range (DLR) and the limit of detection (LOD) were shown in Table 8. LOD was determined in bid stilled water. Eqs. (4) and (5) were used for calculation of enrichment factor and recovery (Rezaee *et al.*, 2006).

$$EF = \frac{C_{sed}}{C_0} \quad (4)$$

Table 8. Quantitative results of DLLME and GC-MS

Linearity range (µg/L)	r^2 [a]	LOD (µg/L) [b]	EF [c]	%R [d]
0.1-50	0.9996	0.02	163.42	63.73

[a] Correlation coefficient

[b] LOD was calculated as the minimum concentration providing chromatographic signals minimum 3 times higher than background noise

[c] Enrichment factor

[d] Extraction recovery

Where EF, C_{sed} and C_0 are the enrichment factor, concentration of analyte in sediment phase and initial concentration of analyte in aqueous sample, respectively

$$ER = \left(\frac{V_{sed}}{V_{aq}}\right) EF \times 100 \quad (5)$$

Where ER, V_{sed} and V_{aq} are the extraction recovery, volume of sediment phase and volume of aqueous sample, respectively.

The performance of DLLME for real samples was tested in tap, well and surface water samples (Tehran, Iran). The results showed that they were free of endosulfan contamination. These samples were spiked with endosulfan standards at 1 µgL^{-1} to assess matrix effects. The results obtained are reported in Table 9. These results demonstrate that matrix effects do not interfere in the quantization process and DLLME-GC-MS may be used as an alternative method for screening organochlorine pesticides in water samples. Fig.5 shows Chromatograms of spiked tap water at different concentrations of endosulfan.The optimized DLLME-GC-MS procedure was compared with SPE-GC-ECD, SPME-GC-ECD, SDME-GC-ECD and SPME-GC-MS (Table. 10). Water extraction and analysis of α- and β-endosulfan is possible with all these methods. In terms of analysis time, SDME and SPME are equilibrium techniques which allow the determination of the target compounds in 20 and 45 min, respectively. However, they are not exhaustive extraction techniques.

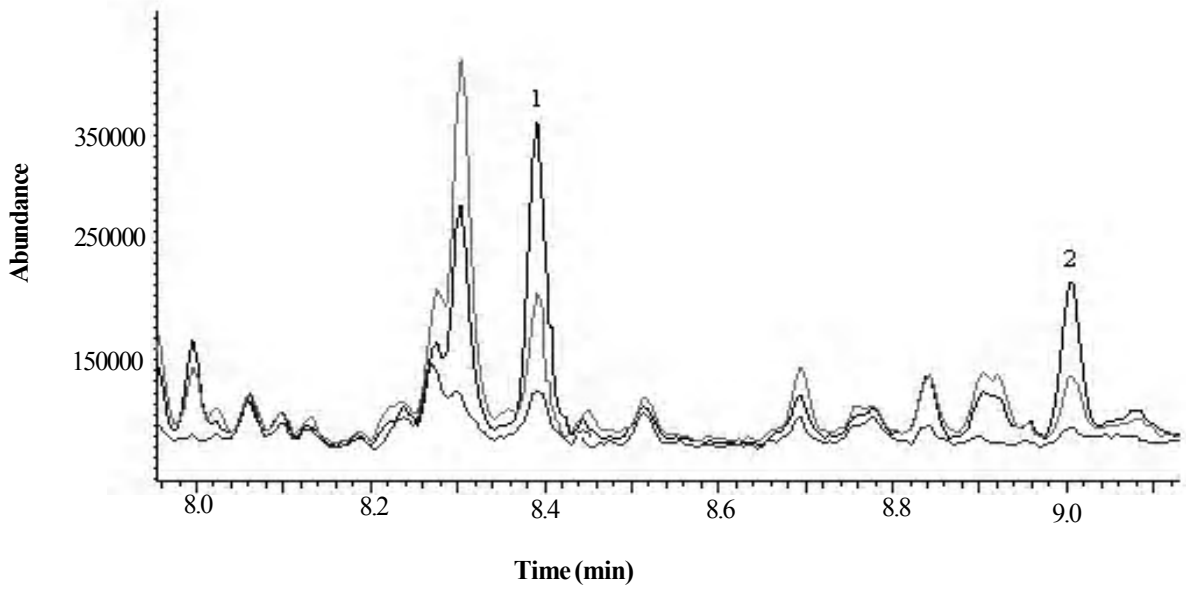

Fig. 5. GC-MS chromatograms of spiked water at 200, 100 and 50 µg/L concentrations of endosulfan. (1) α-endosulfan, (2) β-endosulfan

Table 9. Estimated concentrations and relative standard deviations of endosulfan in spiked tap, surface and well waters at 1.0 µg/L determined by DDLME-GC-MS

Compound	Tap water		Surface water		Well water	
	Mean [a]	%RSD	Mean [a]	%RSD	Mean [a]	%RSD
Endosulfan	1.0	4.7	0.99	8.1	1.1	6.6

[a] n=3

Table 10. Comparison of DLLME with other methods for determination of α- and β-endosulfan in water samples

Extraction techniques	Recovery (%) 0.1 (µg/L)	Linearity range (µg/L)	r^2	LOD (µg/L)	LOQ (µg/L)	Extraction time (min)	Sample volume (mL)	Reference
α-endosulfan								
SPE-GC-ECD	115	0.05–1.0	0.999	0.02	0.04	About 50	100	[2,12]
SPME-GC-ECD	<0.1	0.1–4.5	0.994	0.06	0.13	30	40	[2,12]
SDME-GC-ECD	3.8	0.1–0.9	0.999	0.01	0.02	20	1.8	[12]
SPME-GC-MS	Not reported	0.07–30	0.998	0.01	Not reported	45	3.5	[11]
β-endosulfan								
SPE-GC-ECD	108	0.05–1.0	0.995	0.02	0.03	About 60	100	[2,12]
SPME-GC-ECD	<0.1	0.1–5.0	0.996	0.05	0.10	30	40	[2,12]
SDME-GC-ECD	9.2	0.1–0.9	0.998	0.01	0.03	20	1.8	[12]
SPME-GC-MS	Not reported	0.05–30	0.993	0.02	Not reported	45	3.5	[11]
α- and β-endosulfan								
DLLME-GC-MS	63.7	0.1-50	0.999	0.02	0.1	A few seconds	2	[Represented method]

CONCLUSION

This work indicates that a trace extraction of endosulfan from water samples can be achieved by a DLLME method using experimental design for optimization. This newly developed micro extraction technique provides high recovery and enrichment factor with a much reduced analysis time. Compared to other extraction methods such as LLE, SPE and SPME, this method is simple, rapid, convenient, precise and economical.

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