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Original Article

Development of a Green Single Drop Microextraction Based on Deep Eutectic Solvent and HPLC-UV for Trace Residue Analysis of Three Frequent-Used Pesticides

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Abstract

Background: A green sample preparation method named deep eutectic solvent-based single drop microextraction (DES-SDME) was developed and optimized for determining trace metribuzin, dichlorvos, and fenthion.

Methods: Two hundred seventy experimental runs were performed, and the optimal values of the five influential factors in the DES-SDME method were determined. The design of the study was based on one factor at a time and the peak area of high-performance liquid chromatography was used as a benchmark for comparing analysis results.

Results: After optimizing the effective factors, the linearity range, detection limit and quantification limit of the method were determined by drawing calibration curves for the studied analytes.

Conclusion: The results indicated the success of the developed method in obtaining acceptable figures of merit as a green preparation method with accuracy and precision.

Keywords: Single drop microextraction; Metribuzin; Dichlorvos; Fenthion

Introduction

Pesticides are chemicals that are widely used to kill and keep away any kind of harmful organisms for crops, domestic animals, or humans. The processes of production, formulation, storage, sales, and the widespread use of these chemicals, cause occupational exposure, environmental pollution, and the food contamination (1, 2). Existing studies have shown the occurrence of symptoms, diseases, and irreversible effects on humans and other organisms and damage to the environment due to pesticide exposure. Therefore, monitoring these compounds for control and preventive measures is of great (3, 4).

Metribuzin, dichlorvos, and fenthion are three frequently used pesticides in developing countries (5). Metribuzin is a triazine herbicide used to control weed growth (6). Its long-term persistence in the soil, and high solubility in water can strengthen both its positive effects in pest control and its negative effects on health and the environment (7, 8). Some studies have attributed genotoxicity, nephrotoxicity, hepatotoxicity, neurotoxicity and endocrine toxicity to this pesticide (9, 10).

Dichlorvos is an insecticide from the group of organophosphate compounds (phosphoric acid derivatives) and is also an acetylcholinesterase inhibitor. According to the international agency for research on cancer (IARC) classification, dichlorvos is potentially carcinogenic to humans (2B). Ways to expose to dichlorvos include inhalation, skin contact, and gastrointestinal contact. Due to the high volatility of dichlorvos, the primary way to exposure to it is by inhalation (11).

Fenthion is an organophosphorus pesticide used for a wide range of insects. Phosphorus pesticides kill the insect by suppressing cholinesterase enzymes, disrupting the insect's central nervous system. A common form of exposure to fenthion is occupational exposure that occurs through the skin or inhalation. Another possible route of exposure is through food, especially if the pesticide has recently been used on crops (12).

The stricter standards for food and environmental and occupational exposure limits, on the one hand, and the continuous increase in the production and consumption of pesticides, on the other hand, have increased the importance of developing accurate and reliable techniques for the analysis of these compounds in biological, environmental and food samples (13, 14).

In analyzing chemicals in samples, sample preparation is an essential step. The main objectives of sample preparation are to remove interferences, increase the purity and concentration of the analyte. Reducing the required sample volume, increasing the selectivity of the method, automation, and eliminating or minimizing the use of organic solvents and laboratory equipment are the main goals of sample preparation studies for the development of green methods (15-18).

Unlike the methods with high consumption of solvents, time-consuming, expensive, and low efficiency, that were used in the past for sample preparation (19, 20), recent studies are aimed at developing methods in which the use of organic solvents is eliminated or the minimum possible, and have higher efficiency. For example, in single-drop microextraction (SDME), which is one of the liquid phase microextraction (LPME) techniques (21, 22), a high concentration factor is obtained due to the transfer of analyte from samples with a volume of several milliliters into a droplet with a volume of fewer than ten microliters. By selecting the appropriate solvent, reducing the ratio of acceptor micro-droplet volume to sample, adjusting the conditions and pH of the donor and receiver phases, and using auxiliary reagents in the extraction phase to trap the analyte, the extraction efficiency can be increased. The simplicity of the method, reasonable speed, small sample size, cost-effectiveness, high recycling efficiency, and high enrichment factor are the advantages of this method (23).

The major defect of LPME techniques is using organic solvents as extractor solvents. Toxicity to humans and the environment and not being affordable are the disadvantages of these solvents (24). The proposed solution is to develop a new extraction method using environmentally friendly solvents to remove this defect, which is the most critical limitation of solvent-based microextraction methods.

Deep Eutectic Solvent (DES) is formed by hydrogen bonding between two or more natural substances (25). Features such as being compatible with the environment, non-toxicity or low toxicity, low cost, and the ability to design and manufacture solvents with physicochemical properties according to the needs, make DES superior to other solvents, especially organic solvents (26, 27).

We aimed to develop the green DES-SDME method by substituting deep eutectic solvents instead of common solvents for trace residue analysis of three frequent-used pesticides.

Materials and Methods

This study was conducted at Tehran University of Medical Sciences, Tehran, Iran between 2020 and 2022. Solvents and chemicals used were metribuzin, dichlorvos, and fenthion standards (Sigma-Aldrich, Germany). Hexane, toluene, carbon tetrachloride, acetone, methanol, acetonitrile, phenol, choline chloride, and NaCl with a purity higher than 99%, and buffer solutions (Merck, Germany).

Samples analysis was performed with an HPLC system equipped with a K-1001 pump, a K-2006 UV detector, and a 150 mm length C18 column (Knauer, Germany). The adjusted chromatographic conditions for each of the studied analytes are presented in Table 1. Other equipment's that were used in different stages are: double distillation deionized water machine (PURITE, USA), magnetic stirrer (Chiltern, USA), micro sampler (Socorex, Germany), digital scale (Sartorius, Germany), digital thermometer (TP3001, China), pH meter (Metrohm, Switzerland).

Table 1: The adjusted chromatographic conditions for each of the studied analytes

Variable	*Mobile phase (%)*			*Wavelength*	*Flow rate*	*Injection volume*
Analyte	Acetonitrile	Methanol	Water	(nm)	(mL/min)	(μL)
Metribuzin	-	70	30	290	1	20
Fenthion	70	-	30	250	1.5	20
Dichlorvos	80	-	20	214	1.5	20

Synthesis of DES

Choline chloride (ChCl) and phenol (Ph) as hydrogen bond donor and hydrogen bond acceptor, were combined at 50°C to make the eutectic solvent used in the study. Increasing the molar ratio of phenol decreases the viscosity of the resulting DES. The solution with a 1:1 ratio of choline chloride/phenol was very viscous. Therefore, solvents with molar ratios of 1:2, 1:3, and 1:4, were found to be more suitable for use as extracting solvents in terms of viscosity. The titles DES1, DES2 and DES3 were used for these solvents. The formation of a hydrogen bond is necessary for the synthesis of DES. The formation of this bond can be investigated through Fourier transform infrared (FT-IR) spectrum analysis. For this purpose, the characteristic bonds in the structure of choline chloride (O-H and C-N), phenol (O-H, C=C, and C-O), and their resulting DES were investigated.

Sample preparation procedure

A tiny volume of extractant solvent (in the range of 1 to 6 microliters) attached to the tip of the microsyringe needle should be gently inserted into the sample vial. For this purpose, the microsyringe is placed on top of the sample vial by a support base. Absorption of analyte from sample solution by extracting solvent is called extraction process. Stirring the sample solution increases the speed of reaching equilibrium and thus reduces the extraction duration. Finally, a droplet containing analyte was drawn into the microsyringe and injected into the analyzer to determine the amount. The layout of the used equipment is presented in Fig. 1.

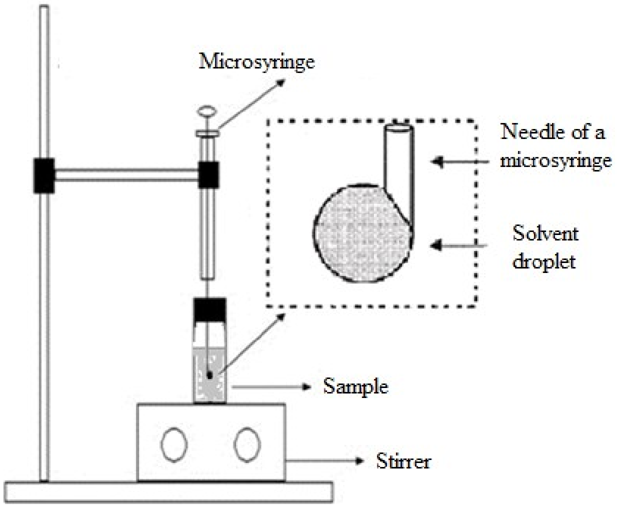

Fig. 1: Layout of equipment used in SDME

Optimization of effective parameters

The type and volume of extracting solvent, ionic strength, pH, and Stirring duration (extraction time), were five effective parameters selected to optimize. When using liquid phase microextraction methods for aqueous phase samples, the chosen extraction solvent should be hydrophobic, capable of forming physical bonds with analytes, and compatible with chromatographic equipment. In order to develop the green DES-SDME method, three DESs with different molar ratios, and three organic solvents, including hexane, toluene, and carbon tetrachloride, were tested, and the relative recovery of the method when using them was compared. The extracting solvent volume as the second important parameter was examined to determine the optimal value. Various volumes of DES1 (1, 2, 3, 4, 5, and 6 μL) were examined. Selecting more volumes for the drop can cause it to fall off the tip of the needle. Increasing the ionic strength of the sample by adding salt helps to transfer the analyte to the extracting solvent. Therefore, different proportions of NaCl (0, 5, 10, 15, 20, 25% W/V) were added to the samples. The pH of the sample determines whether the analyte is in molecular or ionic form. In aquatic samples that have a polar structure, it should be directed towards molecularization to help extract the analyte. Biological samples such as urine, blood, and saliva and environmental samples such as industrial, agricultural, and urban wastewater are aquatic samples. The pH was adjusted to specific values in the range of 2-12, and the relative recovery of the analytes was studied. In the SDME method, the extraction process is done using a magnetic stirrer. During this process, which is known as the extraction time, it is tried to create maximum contact between the extracting solvent drop and the analyte. To determine the optimum level, six durations of 2-12 minutes were investigated.

The optimal values of each parameter were selected through tests at different levels and comparing the results of the levels together; this method is known as "one variable at a time". Thus, in each stage of optimization, four variables were kept constant, and the relative recovery of the analyte from the sample was checked at different levels of one variable. In order to check the analytical performance, calibration curves were drawn. For this purpose, known amounts of metribuzin, dichlorvos, and fenthion were added to blank urine samples diluted with two times the volume of deionized water. The optimized technique was used for the pre-concentration of spiked samples. A total of 270 test steps were performed, and the variables and levels examined for each of them are presented in Table 2. All the optimization steps were performed on the standard sample with a concentration of 1 ppm and a volume of 10 ml.

Table 2: The variables and levels in the optimization steps

Variables	***Levels***					
Type of extraction solvent	Hexane	Toluene	Carbon tetrachloride	DES1	DES2	DES3
Extraction solvent volume (μL)	1	2	3	4	5	6
The amount of added salt (W/V %)	0	5	10	15	20	25
Sample pH	2	4	6	8	10	12
Stirring duration(min)	2	4	6	8	10	12

The optimized method was assayed by Zero (0 μg/L), low (5 μg/L), median (50 μg/L), and high (150 μg/L) concentrations of three studied analytes in the urine samples of non-exposed individuals, and methods intra-day and inter-day precisions were calculated. Also, the RSD% values that show method reproducibility, were calculated. The analysis of the samples without adding standard values was done in order to show that the samples were taken from unexposed people and the analysis results of the spiked samples are exactly related to the standard values added to the samples.

Results

FT-IR

The characteristic bonds in the structure of choline chloride (O-H and C-N), phenol (O-H, C=C, and C-O), and their resulting DES were investigated, and the spectra obtained from their analysis are presented in Fig. 2. Except for the peak related to the O-H bond, which was in the range of 3260 cm-1 in the spectrum of pure phenol and moved to the range of 3236 cm-1 in the spectrum related to the deep eutectic solvent, all the peaks of the two precursors were in the spectrum obtained from DES analysis. This indicates the formation of the expected ChCl-Ph hydrogen bond.

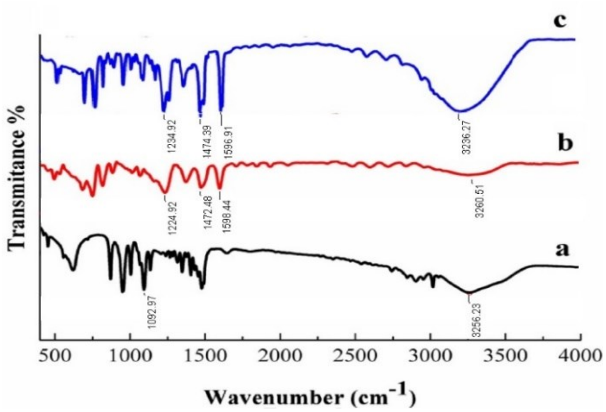

Fig. 2: FT-IR spectra of choline chloride (a), phenol (b) and DES1 (c)

Selection of the optimum levels of effective parameters

Extraction solvent

Among the six studied extraction solvents, in the case of all three analytes, DES1 had the best relative recovery (RR%). The results related to the selection of the optimal extraction solvent are presented in Fig. 3.

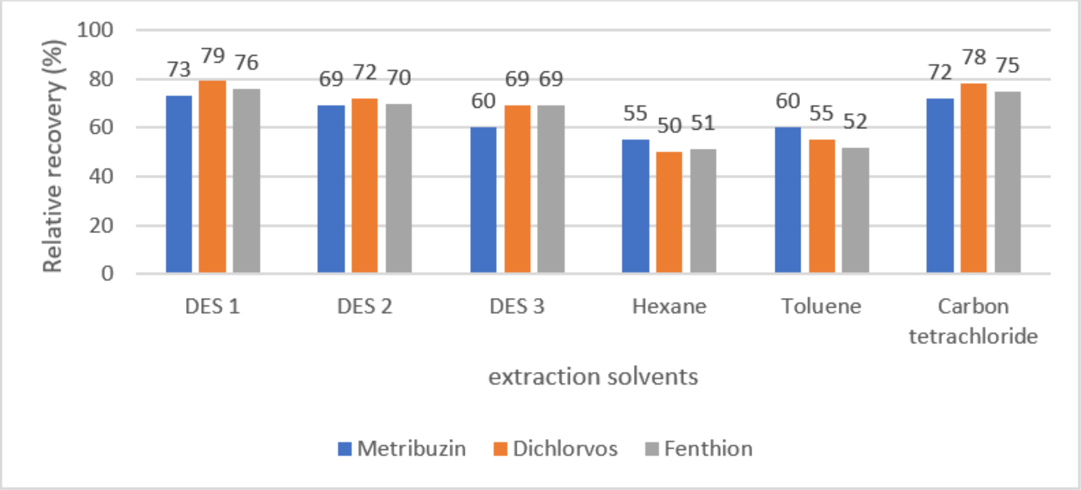

Fig. 3: Results related to the selection of the extracting solvent

The superiority of eutectic solvent in extracting the desired analytes compared to organic solvents, along with the advantages mentioned for DES in the previous section, made DES1 to be selected as the optimal extraction solvent.

Extraction solvent volume

From the volumes of 1 to 4 µL, the relative recovery increased for all three analytes. As shown in Fig. 4a, the amount of recovery did not increase in volumes of 6 and 5 microliters, so the volume of 4 microliters was chosen as the optimal value for the next steps.

Ionic strength

According to the results presented in Fig. 4b, by increasing salt concentration, the relative recovery increased gradually, and the highest recovery for metribuzin, dichlorvos, and fenthion was obtained at a concentration of 15, 15, and 10 W/V%, respectively. At higher concentrations, the relative recovery of the analyte started to decrease due to the increase in sample viscosity. Therefore, the mentioned concentrations were selected as optimal values, and the subsequent optimization steps were performed with these values.

Sample pH

The pH was adjusted to specific values in the range of 2-12, and the relative recovery of the analytes was studied. The highest recovery rate for metribuzin, dichlorvos, and fenthion was obtained at pH=6, 8, and 8, respectively. The results related to this stage are presented in Fig. 4.

Stirring duration

As seen in Fig. 4d, the relative recovery for all three analytes increased in the time intervals from 2 to 6 minutes and was constant in the stirring periods with a duration of more than 6 minutes. Therefore, the optimal stirring duration was 6 minutes for all three analytes.

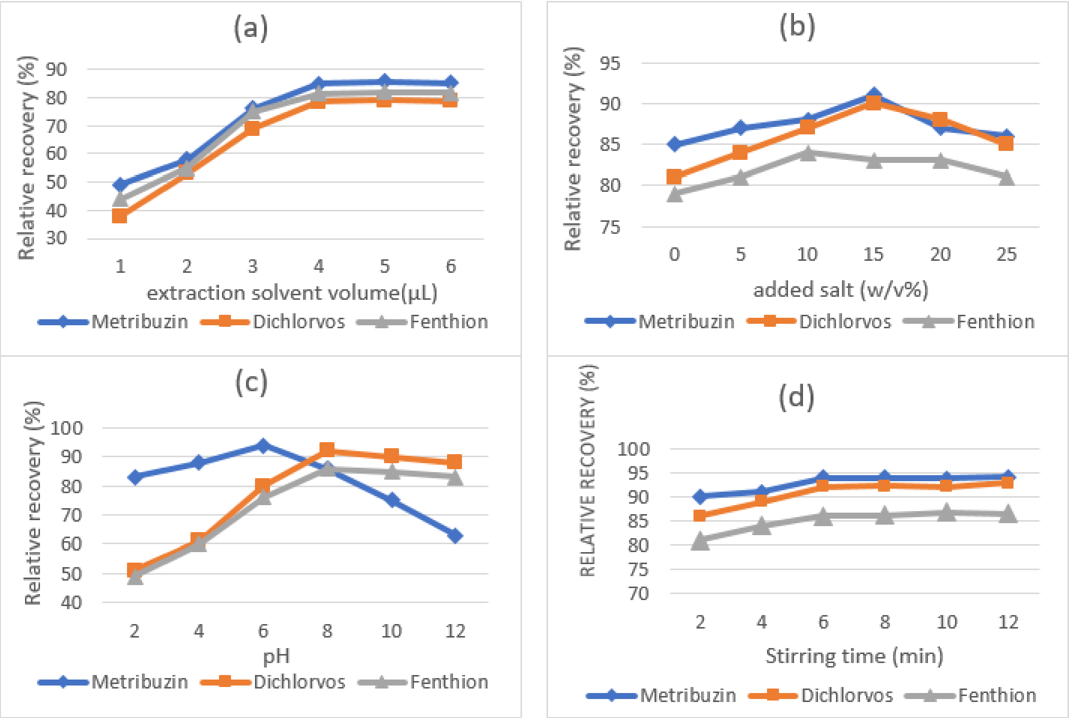

Fig. 4: Effect of extraction solvent volume(a), effect of salt adding (b), effect of pH (c), and effect of Stirring duration (d) on the metribuzin, dichlorvos, and fenthion extraction recovery

Analytical performance

After analyzing the samples and obtaining the results for different concentrations (n=7), calibration curves were drawn, and desirable linearity (r^2=0.999) was obtained in the 5-500 µg/L range. The limits of quantification (LOQ) for metribuzin, dichlorvos, and fenthion were 5, 4, and 4 µg/L. The method detection limit (MDL) amounts were obtained 0.8, 1.1, and 1.1 µg/L. From the obtained analytical figures, it can be concluded that the method developed in the present study has suitable linearity and selectivity for the analyzing three studied analytes.

Reproducibility of the optimized method

According to presented in Table 3, relative recovery values were calculated in the range of 82.7-90.9% in intra-day tests and 80.4-89.7% in inter-day tests. The RSD% values were 3.5% to 7.4%. The obtained results confirmed the validity of the method and the ability to concentrate and purify the desired analytes from a complex biological sample such as urine with high accuracy and precision.

Table 3: RR% and RSD% values in order to assess the reproducibility and repeatability of the optimized method

Real sample	***Spiked levels (µg/L)***	***RR ± RSD (%), (n=6)***					
		Inter-day			Intra-day		
		Metribuzin	Dichlorvos	Fenthion	Metribuzin	Dichlorvos	Fenthion
Urine	0	N.D.*	N.D.	N.D.	N.D.	N.D.	N.D.
	5	88.5 ± 7.4	81.9 ± 6.8	80.4 ± 7.1	89.2 ± 6.2	83.2 ± 6.3	82.7 ± 5.8
	50	89.1 ± 6.5	82.5 ± 5.9	81.1 ± 6.6	89.5 ± 5.1	83.8 ± 5.5	83.6 ± 4.9
	150	89.7 ± 5.1	83.5 ± 5.3	82.3 ± 3.9	90.9 ± 3.5	84.4 ± 4.1	84.1 ± 3.7

*Not detected

Discussion

To check the advantages of the proposed method, a brief comparison was made between the present study and similar studies, presented in Table 4. A number of studies have used organic solvents with a volume of 1000 to 5000 microliters in each step of the work, which may result in several liters if their monthly or annual consumption is calculated.

Table 4: Comparison of the developed sample preparation method to the other sample preparation methods

Sample	***Analyte***	***Method***	***Solvent used***	***MDL (or LOD) (µg/L)***	***LOQ (µg/L)***	***RSD (%)***	***Ref.***
Urine	20 pesticides	DLLME[1], HPLC[2]	100 µL Low transition temperature mixture	0.37	Not reported	3-16	(30)
Urine & Plasma	10 pesticides	DLLME, GC-MS[3]	10 µL DES	2-17	7-57	3-8	(31)
Urine	13 pesticides	SPE[4], LC/MS[5]	1 mL Water/Methanol	Not reported	0.1-13 ng/L	5-27	(28)
Urine	9 pesticides	QuEChERS[6], GC-MS	5 mL, acetonitrile	Not reported	10-20000	4-19	(29)
Urine	3 pesticides	DES_SDME, HPLC	4 µL DES	0.8-1.1	4-5	3.2-7.6	This study

1. Dispersive liquid liquid microextraction
2. High-performance liquid chromatography
3. Gas chromatography with mass spectrometry
4. Solid phase extraction
5. Liquid chromatography with tandem mass spectrometry
6. Quick, Easy, Cheap, Effective, Rugged and Safe

These solvents are toxic to laboratory personnel and the environment (28, 29). In the current study, green deep eutectic solvent, which does not have the disadvantages of organic solvents, was used with a much smaller volume (up to 1000 times less). In addition, even though the present method requires less equipment and chemicals compared to other studies (30, 31), and as a result, less cost and does not have their complexity, the comparison of RSD, MDL, and LOQ values showed that it has high sensitivity, accuracy and reproducibility.

Conclusion

Considering the complex composition of biological samples, the presence of small amounts of chemicals in the samples, the reduction of permitted amounts and new standards, and the importance of issues related to the sustainability and greenness of laboratory methods; It is necessary to develop sample preparation methods that can cover these issues. As the main result of the present study, DES-SDME as a green sample preparation method was successfully developed by optimizing the extraction process on various influencing variables and substituting deep eutectic solvents instead of conventional solvents. Due to the satisfactory performance shown in this study, the deep eutectic solvent can be a suitable option for future studies as an alternative to toxic organic solvents in analytical methods. The advantages of the method developed in the present study include the need for less equipment, chemicals, skill, and cost, which makes it a sustainable and green method. Therefore, it can be used in laboratories without worrying about having harmful effects on humans and the environment.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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