Optimization of dispersive liquid–liquid microextraction procedure for detecting chlorpyrifos in human urine samples

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Abstract
Background: Selecting an effective sample preparation method to measure target pesticides in biological matrices is a serious challenge for researchers. This study aimed to optimize the dispersive liquid-liquid microextraction (DLLME) technique to obtain a simple, valid, and fast method with high efficiency to detect chlorpyrifos in urine samples.

Methods: DLLME, coupled with high performance liquid chromatography equipped with ultra violet detector, was used to extract chlorpyrifos pesticide in human urine samples. Different affecting parameters on the efficiency of the method were optimized using one factor at a time method.

Results: The limit of detection and enrichment factor of the method was 0.5 and 230 µg L-1, respectively. Linear calibration curve with 1-500 µg L-1 concentration range was used. The relative standard deviation (RSD) for 6 replicate experiments at the concentration of 200 µg L-1 was less than 5%. The relative recoveries of spiked urine samples were 96.3%, 102.3%, and 98.7% at 3 different concentration levels of 50, 200, and 1000 µg L-1, respectively.

Conclusion: Compared to other extraction techniques, the optimized DLLME resulted in some advantages such as shorter extraction time, high extraction efficiency, and good enrichment factor for the extraction of chlorpyrifos from human urine samples.

Keywords: Chlorpyrifos, Dispersive liquid-liquid microextraction, Urine, HPLC

Introduction

Organophosphate pesticide poisoning is commonly found among toxin-producing workers in factories, toxin spraying workers, and consumers of contaminated foods and equipment (1, 2).

Most farmers who use pesticides daily are at risk due to the lack of proper knowledge about poisoning of these chemicals and correct ways of working with them (3). Therefore, it is necessary to develop appropriate techniques to extract these hazardous compounds from biological samples to evaluate the exposure of workers.

Conventional sample preparation techniques, such as liquid-liquid extraction (LLE), solid-liquid extraction

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↑What is "already known" in this topic:
Most sample preparation techniques have been used in combination with gas chromatography as a high selective or sensitive detection system, which are expensive and cannot be used widely for the analysis of organophosphorus pesticides in developing countries.

→What this article adds:
The proposed method in this study, without any special detector, is a sensitive, simple, rapid, and repeatable sample preparation method and can be used for the extraction and preconcentration of chlorpyrifos residues from aqueous samples.

(SLE), and soxhlet extraction, are widely accepted and used for routine applications. Furthermore, new studies are being conducted in this field with the aim of developing faster and stronger preparation and extraction techniques (4-7). Solvent microextraction (SME) is a method for preparing samples by extraction and condensation of liquid, gas, and solid samples using 100 µL solvent or less. The SME is used to extract, purify, and concentrate the volatile, nonvolatile, polar, nonpolar, ionic, and metallic analytes from environmental, biological, and agricultural samples. There are several types of SMEs that are generally divided into 2 major categories: (1) exposed to solvent, and (2) solvent with membrane (7-9)

Dispersive liquid-liquid microextraction (DLLME), hollow-fiber microextraction (HFME), single-drop microextraction (SDME), and liquid-liquid microextraction (LLME) were the main and most widely used methods of the SME procedure in recent years. Unlike other sample preparation methods, SME is still used for scientific research (10-13).

DLLME, which belongs to the category of exposed solvents, is widely used as a sample preparation method with high extraction recovery (14-17). Extracting solvent, insoluble in water, is dissolved in dispersive solvent, then, it is rapidly injected into the liquid sample by a proper syringe. The rapid injection of a mixture of organic solvents into the water causes the insoluble solvent to be extracted rapidly in small microdrops from the target analyte. The enriched organic phase is separated from the liquid sample by centrifuging and it is directly injected to the analyzing instrument (18-21).

Chlorpyrifos (trade name of Dursban EC40.8%), as a subgroup of organic phosphorus insecticides and phosphorothioic acid is used to control a wide range of agricultural and domestic pests (17). This pesticide has nonsystemic, gastro-intestinal, and respiratory effects (22-25). The durability of this compound in the soil is 2 to 4 months, and its toxicity is very high for humans and animals. The ability of this poison to inhibit cholinesterase enzyme in the nervous system has attracted much attention to control and reduce workers' exposures (26-29).

The present study aimed to optimize DLLME as a fast, effective, low-cost, and simple extraction method to determine the trace amounts of chlorpyrifos in human urine samples. In this study, a useful sample preparation method, with high extraction of very few pesticide residue in the matrix, was developed.

Methods
Reagents and solutions
Chlorpyrifos with purity of more than 98% were provided by Dr. Ehrenstorfer Company (Germany). Organic solvents, including carbon tetrachloride, carbon disulfide, chloroform, methanol, acetonitrile, and acetone, were purchased from Merck (Darmstadt, Germany). Analytical-reagent grade sodium chloride, hydrochloric acid, and sodium hydroxide were also obtained from Merck. Deionized water was purchased from Behan Company (Tehran, Iran). A stock solution of chlorpyrifos (1000 ppm) was prepared by dissolving an appropriate amount of the pesticide in acetonitrile. Working standard solutions were prepared daily by diluting the stock solution with deionized water.

Instrumentation
HPLC (HPLC pump k-1001, UV detector k-2600; Knauer, Japan), equipped with a UV detector, was used to determine and separate chlorpyrifos. The separation was performed on Agilent Eclipse Plus C18 column (L= 250 mm, ID = 4.6 mm; Reprosil-PUR C-18 AQ 10 µm) using methanol–water solution (60:40, v/v) as mobile phase. The pump flowrate and column temperature were set at 1.5 mL/min and 25°C, respectively. The chromatographic response for the analyte and matrix interference was acceptable under the detection wavelength of 203 nm. A Hettich zentrifugen Rotofix 32 (Baoding, China) was used for centrifugation. The samples were ultrasonically irradiated in water bath at 150 W and 40 kHz using an ultrasonic equipment (SonoSwiss SW 6 H). All glassware used in the experiments were washed with acetone and deionized water and dried in an oven at 50°C temperature.

Dispersive liquid–liquid microextraction procedure
First, 10 mL spiked urine sample with defined concentration of analyte (1 ppm) was poured into a 15mL centrifugal tube; then, 1.5 mL of methanol containing 150 µL carbon tetrachloride was quickly injected to the centrifugal tube. The cloudy solution was centrifuged for 5 min at 4000 rpm and the extractant was settled to the bottom of centrifugal tube. The phase containing chlorpyrifos was separated by a syringe and poured into another test tube, and its solvent evaporated under the gentle flow of N_2. Finally, the remaining settled phase was dissolved in methanol and 20 µL of it was withdrawn using a 100 µL microsyringe and injected into the HPLC for quantification (Fig. 1).

Eight factors that could potentially affect the chlorpyrifos extraction were examined to find their optimum levels. These factors included the extraction solvent, disperser solvent, volume of the extraction solvent, volume of the disperser solvent, centrifugation time and speed, salt addition, and sample pH. In each step, 7 factors were constant and 1 varied in different levels to determine the optimum quantity. Figure 2 shows chromatograms of aqueous sample of 1 ppm chlorpyrifos before (A) and after (B) of ap-
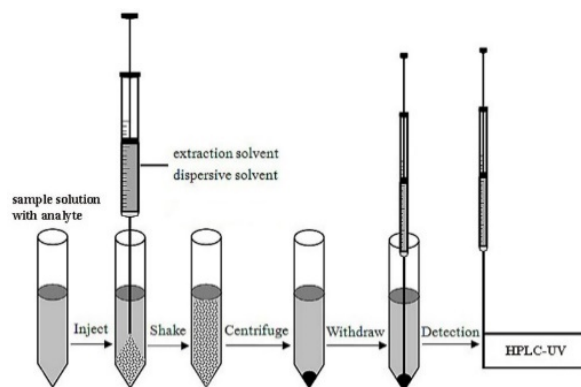

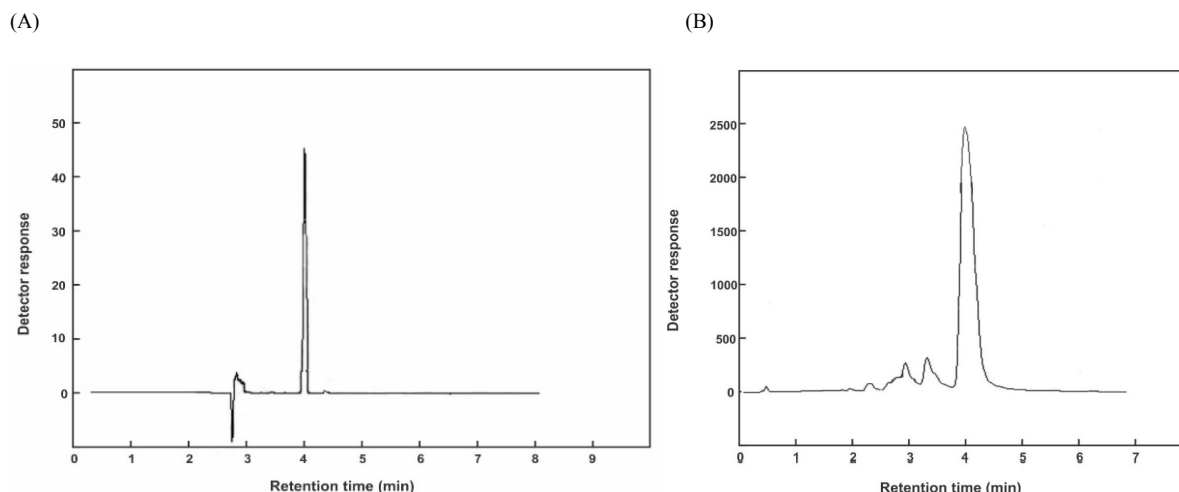*Fig. 1.* The principle of DLLME method

(A)

(B)

Fig. 2. Chromatograms of aqueous sample of chlorpyrifos before (A) and after (B) preconcentration

plying DLLME procedure.

Urine sample preparation

Urine samples were collected from exposed workers and stored in a freezer at−18°C. Urine samples (5.0 mL) were placed in centrifuge tubes and diluted with 50 mL double-distilled water. PH was adjusted at 6 by adding sodium hydroxide solutions to the samples. Next, the prepared specimen was analyzed according to the proposed preparation method.

Enrichment factor and extraction recovery

To develop DLLME method for preconcentration of chlorpyrifos, some parameters controlling the extraction efficiency were investigated using sample solutions with the analyte concentration of 1 ppm. To evaluate the extraction efficiency, enrichment factor (EF) and extraction recovery (ER) of the analyte were calculated by the following equations:

$$EF = C_{sed} / C_0 \tag{1}$$

Where, C_{sed} and C_0 are the concentrations of the analyte in the settled phase and in the aqueous samples before extraction, respectively.

C_{sed} was obtained from the calibration curve of direct injection of standard solutions.

$$ER= C_{sed}V_{sed}/C_0V_{aq}\times100\%= EF\times(V_{sed}/V_{aq})\times100\% \tag{2}$$

Where, V_{sed} and V_{aq} are the volumes of the settled phase and the aqueous sample, respectively. The average of 3 replicate extractions was reported for all experiments.

Results

Effect of extraction solvent

An extraction solvent was selected based on capability to extract the analyte as well as its appropriate chromatographic behavior. Three solvents, including carbon tetrachloride (CCl₄), carbon disulfide (CS₂), and chloroform

(CHCL₃), were examined as extraction solvents in DLLME. The important properties of selected solvents, such as density and solubility, could affect the extraction efficiency of the target analyte. For this purpose, 10 mL aqueous solutions of chlorpyrifos (1 ppm) were used to optimize the extraction solvent. According to the obtained results, no distinct cloudy solution was formed using CS₂ and CHCL₃ as extraction solvents, indicating that they could not effectively disperse among aqueous sample solution because of low extraction capability. Contrasted with CS₂, CHCL₃, CCl₄ resulted in the highest extraction efficiency for chlorpyrifos. Hence, CCl₄ was selected as the optimum extraction solvent for subsequent experiments.

Effect of disperser solvent

The type of disperser solvent is very important for obtaining preconcentration of the analyte. The chosen solvents must be appropriately miscible in both extraction solvent and sample solution, so that they can form a distinct cloudy solution. Therefore, 4 possible disperser solvents, including methanol, ethanol, acetonitrile, and acetone, were examined. Methanol showed the highest extraction recovery for the analyte compared to other mentioned solvents. Taking into account the data, methanol was selected for later experiments.

Effect of volume of extraction solvent

The effect of extraction solvent volume on the enrichment factor and extraction recovery of the analyte was evaluated by using 2 mL methanol containing different volumes of CCl₄ (50, 100, 150, and 200μL). With the increase of the CCl₄ volume, the extraction recoveries of chlorpyrifos increased. It was also found that, the volume of the sediment phase at the bottom of the test tube increased by elevating the volume of CCl₄ from 50 to 150μL. According to the results, more volume of CCl₄ led to the highest extraction recovery and after that it was constant. Therefore, the volume of 150μL was selected as the optimal volume of CCl₄.

Effect of volume of disperser solvent

The other parameter that could affect the extraction recovery and enrichment factor was the volume of disperser solvent. Different methanol volumes (0.5, 1, 1.5, 2, 2.5 mL) containing 150 µL CCl₄ were used to find the optimal volume. The extraction recoveries of the analyte increased at first and then decreased by raising the volume of methanol. At low volumes of methanol, the cloudy solution was not formed completely, leading to the low extraction recovery of the analyte. When the volume of methanol increased to 1.5 mL, the highest extraction recovery and enrichment factor was obtained due to decreasing the solubility of CCl₄ in aqueous solution. However, the extraction recovery decreased when the volume of methanol was more than 1.5 mL. This decrease can be explained with the increase in the ratio of the dispersive solvent to the extraction solvent and prevention in settling the extraction solvent.

According to the results, 1.5 mL methanol was chosen to obtain high enrichment factor and extraction recovery.

Effect of centrifugation time and speed

To separate the extractant phase, an important factor in DLLME is centrifugation process. This step destroys the cloudy solution and helps the extraction solvent to settle at the bottom of the tube. The effects of centrifugation time and rate on the extraction efficiency were examined and optimized in the ranges of 5–20 min and 2500-4000 rpm, respectively. According to the results, the time of 5 minutes and the speed of 4000 rpm were selected as the optimum levels of centrifugation for next experiments.

Effect of salt addition

To prove the effect of ionic strength on the extraction efficiency, different concentrations of sodium chloride (0, 2, 4, and 6% w/v) were investigated. Salting out can decrease the solubility of the analyte in the aqueous phase and increase extraction into the organic phase. Based on the obtained results, increasing the NaCl concentration led to the lower extraction efficiency, as the increase of the aqueous phase viscosity decreased the diffusion coefficient of the analyte. Therefore, next experiments were performed with no salt addition.

Effect of sample pH

Sample pH is also another important factor affecting the extraction efficiency. By adding the appropriate amount of hydrochloric acid or sodium hydroxide solutions to water samples, the stability of chlorpyrifos under the pH range of 2–10 was tested. It was indicated that the higher extraction recovery of the analyte was obtained at pH 6. Therefore, doubled-distilled water was used without pH adjustment in the study.

Analysis

Analytical features of the method: The analytical characteristics of the method, including linear range (LR), limit of detection (LOD or MDL), limit of quantification (LOQ), correlation coefficient (r^2), relative standard deviation (RSD%), enrichment factor (EF), and efficiency recovery (ER), were determined under the optimized conditions to evaluate the performance of the method. The obtained results were summarized in Table 1. linearity was over a broad concentration range for the pesticide, with correlation coefficient (r^2) of 0.993. The MDL and LOQ, calculated based on signal to noise ratio (S/N) of 3 and 10, were 0.5 and 5, respectively. The RSD values were less than 5% for interday and intraday precisions, indicating an acceptable repeatability for the developed method. The EF and ER for the pesticide were 230 and 95.7%, respectively. Satisfactory repeatability, high EF and ER, and low MDL and LOQ are the main advantages of the proposed method.

Urine sample analysis: The proposed method was applied to the preconcentration and determination of chlorpyrifos in the spiked urine samples. To validate the accuracy of the DLLME procedure, samples were spiked with the target analyte at 3 different concentration levels of 50, 200, and 1000 µg L⁻¹ and analyzed in triplicate using the recommended method. The analyte recoveries are shown in Table 2. According to obtained data, the relative recoveries ranged from 96.3%-102.3%. The relative recoveries of the analyte indicated no significant differences in concentration levels of 50, 200, and 1000 µg L⁻¹, confirming the validity of the proposed method. The obtained RSDs for the real samples were fairly low at different concentrations. These results indicate that the real sample matrix has no significant effect on the proposed method for preconcentration of chlorpyrifos from urine sample.

Discussion

The comparison of LR, RSD, MDL, LOQ and EF obtained for the presented method with those of other reported methods for analysis of the target analyte in different samples is summarized in Table 3. The RSD of the proposed method is comparable or better than those reported for the other methods. This study found lower MDL and LOQ compared to most reported methods. The MDL was

Table 1. Quantitative features of the proposed method for chlorpyrifos

LR (µg L⁻¹)	r^2	MDL (µg L⁻¹)	LOQ (µg L⁻¹)	RSD (%) 200 µg L⁻¹ spiked (n=6)		EF	ER (%)
				Intra-day	Inter-day		
5-500	0.9931	0.5	5	1.9	4.69	230	95.7

Table 2. Relative recovery (RR) and RSD values of chlorpyrifos in urine sample

Spiked levels (µg L⁻¹)	RR (%)	RSD (%) (n=3)
50	96.3 ± 2.1[a]	2.2
200	102.3 ± 4.2	4.07
1000	98.7 ± 3.1	3.1

[a] Mean of three determinations ± standard deviation

Table 3. Comparison of the presented method with other methods used in the analysis of the target analyte

Pesticide	Sample	MDL	LOQ	RSD (%)	LR	Extraction Factor	Extraction time (min)	Method	Reference
Diazinon	Water	1.4 µg L^{-1}	-	9.4	5-500 µg L^{-1}	-	20	PN–SDME– GC–MS	(30)
Chlorpyrifos	sample	1.6 µg L^{-1}	-	8.3	5-500 µg L^{-1}				
Malathion		1.6 µg L^{-1}	-	7	5-500 µg L^{-1}				
Diazinon	Aqueous	0.65 µg L^{-1}	2.2 µg L^{-1}	6	3-40000 µg L^{-1}	-	15	MWA– DLLME– GC–FID	(31)
Chlorpyrifos	sample	0.74 µg L^{-1}	2.5 µg L^{-1}	4	3-40000 µg L^{-1}				
Malathion		1.3 µg L^{-1}	4.5 µg L^{-1}	4	3-40000 µg L^{-1}				
Diazinon	Summer crops	2 µg kg^{-1}	6.3 µg kg^{-1}	5.5	5-500 µg kg^{-1}	50	35	UASE– DLLME– SFO– HPLC– UV	(32)
Chlorpyrifos		4 µg kg^{-1}	11.8 µg kg^{-1}	9.1	10-800 µg kg^{-1}				
Diazinon	Fruit juice	0.5 µg kg^{-1}	-	1.51	4-200 µg kg^{-1}	50	40	CPE–GC– FPD	(33)
Chlorpyrifos		1 µg kg^{-1}	-	2.27	4-200 µg kg^{-1}				
Malathion		1 µg kg^{-1}	-	1.88	4-200 µg kg^{-1}				
Chlorpyrifos	Urine	0.5 µg L^{-1}	5 µg L^{-1}	4.07	5-500 µg L^{-1}	230	4	DLLME– HPLC–UV	This work

lower or comparable than most of the mentioned techniques even those employing mass spectrometry (MS) detectors. Most of the mentioned techniques have used a high selective or sensitive detection system, such as flame photometric detection (FPD), flame ionization detector (FID), or MS; and all of these detectors in combination with gas chromatography (GC) are expensive and cannot be widely used to analyze organophosphorus pesticides in developing countries. The proposed method in this study does not need any special detector, but it still is sensitive, simple, rapid, and repeatable and can be used for the extraction and preconcentration of chlorpyrifos residues from aqueous samples.

In a study done by Wang et al in 2011 (30), pneumatic nebulization single-drop microextraction method was used to determine organophosphorus pesticides by gas chromatography–mass spectrometry. The results showed that MDL and RSD were 1.6 µg L^{-1} and 8.3, respectively. However, in the present study, MDL and RSD were 0.5 µg L^{-1} and 4.07, which were much lower. Also, in studies conducted by Farajzadeh et al in 2016 (31), ionic liquids that are lighter than water were used as extraction solvents and achieved favorable and very similar results in the present study.

In this study, DLLME procedure was successfully developed from aqueous samples for the extraction of chlorpyrifos. The most important effective factors for the extraction of the analyte were investigated and optimized. The proposed procedure had some advantages in comparison with other extraction techniques such as shorter extraction time, better reproducibility, and higher enrichment factor. Also, good precision, suitable recoveries, broad dynamic linear range, and low limit of detection were attained using the DLLME method.

Conclusion

In the present work, DLLME procedure was successfully optimized for the extraction of chlorpyrifos from human urine samples. Different effecting factors in extraction of the analyte were investigated and developed. The proposed optimized procedure in comparison with the other extraction techniques had more advantages such as shorter extraction time, better reproducibility, and higher enrichment factor. The worthy analytical figures, such as

suitable recoveries, good precision and enrichment factor, wide dynamic linear range, and low limit of detection, were attained by DLLME method. In conclusion, according to obtained results, the proposed method can be used as a simple procedure to provide high preconcentration efficiency to determine chlorpyrifos in complex matrices.

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