Research Article

Development and Validation of a Simple and Rapid HPLC Method for the Evaluation of Pesticide Residues in Plasma Samples of Farmers; Application in Toxicological and Risk Assessment Studies

Mir Ali Farajzadeh[1,2*] , **Ali Mohebbi[1]**, **Mohammad Reza Afshar Mogaddam[3,4]**

[1]Department of Analytical Chemistry, Faculty of Chemistry, University of Tabriz, Tabriz, Iran.
[2]Engineering Faculty, Near East University, 99138 Nicosia, North Cyprus, Mersin 10, Turkey.
[3]Food and Drug Safety Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[4]Pharmaceutical Analysis Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Article Info

Abstract

Background: In the present work, an analytical method based on high performance liquid chromatography–diode array detection has been reported to evaluate the presence of some widely used pesticides in plasma samples of the farmers exposed to the pesticides in farm lands and rural zones. Prior to instrumental analysis, the analytes are extracted using a two–step extraction procedure based on a combination of cold–induced homogenous liquid–liquid extraction and dispersive liquid–liquid microextraction.

Methods: In this method, initially acetonitrile is added to a tube containing the plasma sample and the mixture is vortexed. By this action, the proteins of plasma are precipitated at the bottom of the tube after centrifugation and a homogenous solution is collected on them. Subsequently, the mixture is placed in liquid nitrogen for a few seconds to freeze the aqueous phase. As a result, the homogenous state is broken and the analytes are extracted into the supernatant organic phase (acetonitrile), which is subsequently removed and used in the following microextraction method.

Results: Under the optimum extraction conditions, the proposed method indicated good extraction recoveries (59–80%), satisfactory repeatability (relative standard deviation ≤ 6% for intra– and inter–day precisions), and low limits of detection (1.4–2.3 µg L^{-1}).

Conclusion: Finally, various plasma samples of the farmers were analyzed by the introduced method. Ease of operation, being environmentally friendly, rapidity, and low cost can be the main advantages of the introduced approach.

Introduction

All over the world, the use of pesticides is the primary choice of farmers to control pests and plant pathogens in order to obtain high quantity and quality of agricultural productions. Despite the mentioned merits of the pesticides, they can have adverse effects on the environment and human health.[1] Pesticides can be absorbed in human body by their ingestion from food and drinking water, inhalation from air, and dermal contact.[2] Farmers are a group of population who directly exposed to pesticides in the greenhouses and agricultural lands while mixing, handling, and spraying of them.[3] Pesticides associated health problems manifest as a series of symptoms depending on severity of exposure. The mild poisoning with pesticides can cause several problems such as nausea, headache, and dizziness, while the chronic toxicity can lead to serious difficulties like hormone disruption, cancer, suppressive effect on the immune system, and brain damage.[4] Therefore, the detection and determination of pesticide residues in biological fluids of farmers is of great importance. Determination of pesticides is usually performed by means of chromatographic techniques, either gas chromatography[5,6] or high–performance liquid chromatography (HPLC).[7,8] Despite the significant progresses in analytical techniques still the direct analysis of most samples is not feasible because of the matrix complexity of samples, especially biological fluids, and/or low concentration of the analytes. So, an extra step named as sample preparation is needed to achieve the necessary levels of sensitivity, enrichment, and clean–up.[9,10] Liquid–liquid extraction as a conventional mode of sample pretreatment procedure has been extensively used for the extraction of various analytes from aqueous solutions despite its disadvantages such as requirement of numerous time–consuming and laborious processes, and consumption of large volume of toxic solvents.[11]

Homogeneous liquid–liquid extraction (HLLE) is another approach that has attracted a great deal of attention from researchers since it eliminates the use of time–consuming process and large volumes of organic solvents. In this method, a water–miscible extraction solvent, mostly acetonitrile (ACN), is mixed with an aqueous sample solution containing the analytes to form a homogenous solution which is subsequently broken by adding a phase separation agent.[12] In an ACN–water based HLLE, the homogeneous solution can be separated into two phases with the addition of phase separation agents such as sugars [13] and salts[14] or cooling the solution.[15] In the case of the last mode, at low temperatures (< –20 °C) the solubility of ACN in the aqueous phase decreases and ACN phase containing the analytes is separated from the aqueous solution as the upper layer. In this mode unlike the other modes, there is no requirement to use of any foreign inducer material to break the homogenous state that can decrease the cost of analysis. Up to now, this method was utilized for the clean–up, extraction, and preconcentration of analytes in samples such as milk,[16] sticky traditional Chinese patent medicine,[17] meat,[18] and tea.[19] The major problem of these methods is the time–consuming cooling procedure (takes time between 1 to 12 h).[16–19] Also, there was no report concerning its application in a complex matrix e.g. plasma. The purpose of this work was to introduce a sample preparation approach based on the combination of cold–induced HLLE and dispersive liquid–liquid microextraction (DLLME) for the extraction and preconcentration of some pesticides in plasma samples of farmers prior to their quantification with HPLC–diode array detection (DAD). Unlike most of the published papers, in this study, there is no need for addition of any foreign substance or dilution of the plasma sample (in order to reduce the matrix effect). Also, precipitation of plasma proteins and extraction procedure are done in one step. ACN acts as a precipitation agent for proteins, an extraction solvent, and a dispersive solvent simultaneously. It leads to minimize the use of organic solvents and indicates a great merit in the era of green chemistry. Ease of operation, being environmentally friendly, rapidity (phase separation is occurred in a few seconds using liquid nitrogen), and low cost can be the main advantages of the introduced approach.

Materials and Methods
Chemicals and solutions
Metalaxyl, acetamiprid, clodinafop–propargyl, oxadiazon, chlorpyrifos, penconazole, fenoxaprop–P–ethyl, haloxyfop–R–methyl, and hexaconazole with purity higher than 98% were purchased from Dr. Ehrenstorfer (Augsburg, Germany). HPLC–grade methanol, water, and ACN were supplied from Merck (Darmstadt, Germany). Chloroform, carbon tetrachloride, and dichloromethane were also from Merck. 1,1,1–Trichloroethane (1,1,1–TCE) was from Janssen (Beers, Belgium). Sodium chloride was purchased from Dr. Mojallali Company (Tehran, Iran). A stock solution of the analytes (100 mg L^{-1}, each pesticide)

was prepared in methanol. This solution was daily diluted with HPLC–grade water at appropriate ratios and used as working standard solutions.

Apparatus
The HPLC analysis were conducted using a Hewlett–Packard 1090–II liquid chromatograph (Palo Alto, CA, USA) equipped with a DAD. All injections were performed manually using a 10–µL sample loop. Chromatographic separation of the analytes was carried out on a SUPELCOSIL™ LC–18 HPLC column (15 cm × 3 mm i.d., particles size of 3 µm) (Supelco, Bellefonte, PA, USA). A mixture of methanol–water (80:20, v/v) at a flow rate of 0.25 mL min^{-1} was utilized as the mobile phase. Monitoring of the analytes was done at 212 nm. The column temperature was thermostated at 45 °C. A Universal 320 R centrifuge (Hettich, Kirchlengern, Germany) and a vortex mixer (Velp Scientific, Italy) were used for accelerating phase separation and vortexing, respectively.

Samples
A pesticide–free plasma was obtained from Blood Transfusion Organization (Tabriz, Iran) and kept frozen at –20 °C until its use as a blank. Five distinct blood samples were obtained from five male farmers (32–53 years old) exposed to the pesticides in the farm lands and rural zones. The blood sample was placed in a citrate treated plastic test tube and centrifuged for 10 min at 4000 rpm. In order to remove blood platelets, the supernatant phase was removed and centrifuged at 9000 rpm for 5 min. The obtained plasma was used in the following extraction procedure. The volunteers were properly informed about the study and they signed written consent forms.

Extraction procedure
A 1.0 mL of the blank plasma spiked with the pesticides at a concentration of 250 µg L^{-1} (each pesticide) or plasma sample was transferred into a 10–mL glass test tube. Then, 1.5 mL ACN was added and the mixture was vortexed for 2 min. By this action the proteins of plasma were precipitated at the bottom of the tube and a homogenous solution was obtained on them. Subsequently, the tube was placed in the liquid nitrogen for 10 s. By doing so, the homogenous state was broken and a two–phase system was formed (aqueous phase plus the precipitated proteins were frozen at the bottom of the tube while ACN was collected as a liquid phase on them). After that, 1.0 mL of the collected ACN phase was removed, mixed with 70 µL 1,1,1–TCE, and injected into 5 mL HPLC–grade water. After centrifugation at 4000 rpm for 4 min, the whole of the sedimented organic phase (50 µL) was transferred into a home–made microtube (42 × 7 mm) using a 250–µL microsyringe and evaporated to dryness under stream of nitrogen at room temperature. The residue containing the enriched analytes was reconstituted in 20 µL of the mobile phase and injected into HPLC system for the quantitative analysis (Figure 1).
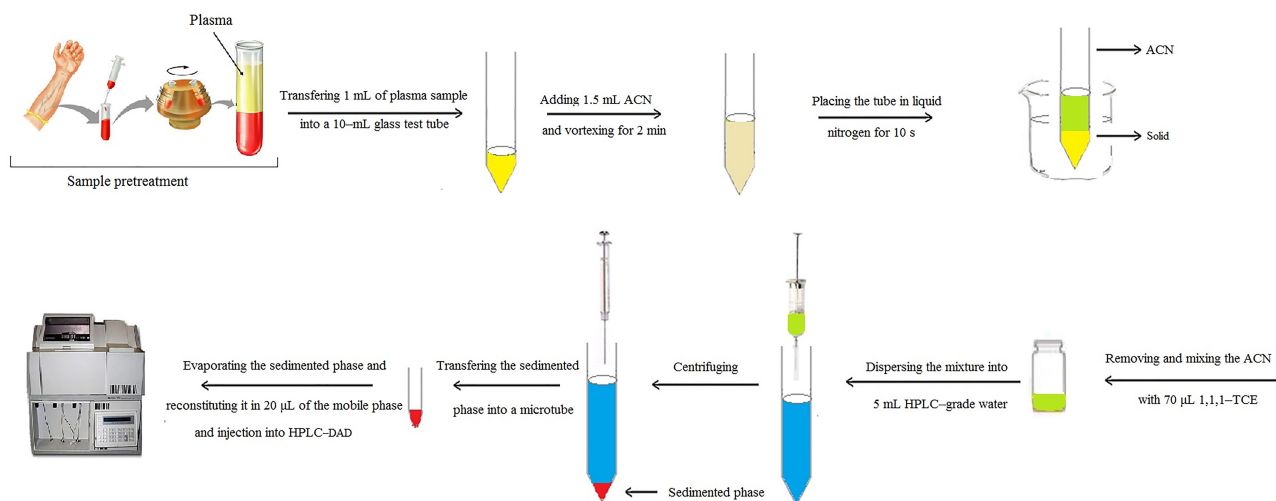
Figure 1. Extraction and preconcentration procedure.

Calculation of extraction recovery (ER)

The percentage of the total analyte amount (n_0) that is transferred into the final phase (n_{fin}) is defined as ER. The final phase was 20 μL mobile phase that used for dissolving the residue after evaporation of the sedimented phase.

$$ER\% = \frac{n_{fin}}{n_0} \times 100 = \frac{C_{fin} \times V_{fin}}{C_0 \times V_s} \times 100$$

where C_{fin} and C_0 are the analyte concentration in the final phase and the initial concentration of the analyte in plasma, respectively. Also, V_{fin} and V_s are the volumes of the final phase and plasma, respectively.

Results and Discussion
Optimization of HLLE parameters
Study of ACN volume

According to the previous reports among the common solvents such as acetone, ACN, and methanol, used in HLLE only ACN forms a two–phase system with an aqueous solution upon lowering the temperature.[15,18] Therefore, it was selected as an extractant in the present study. It is noted that, in the first step of the present work, ACN acts as a precipitation agent for proteins and in the second step (DLLME), it plays the role of a disperser. The volume of ACN can affect the extraction efficiency of the method and should be optimized. To evaluate this parameter, different volumes of ACN (0.5–2.5 mL at 0.5–mL intervals) were studied. Based on the results, in volume of 0.5 mL ACN, no phase was separated by cooling the solution and the approach became useless. In the cases of 1.0, 1.5, 2.0, and 2.5 mL, the volume of the separated ACN phase was 0.7, 1.1, 1.6, and 2.0 mL, respectively. In all cases, 1.0 mL of the separated phase was removed and utilized in the following DLLME, except in the case of 1.0 mL ACN in which the collected phase volume was 0.7 mL. In this case, all of the separated phase was removed and mixed with 0.3 mL pure ACN. Considering the results (Figure 2), the highest ERs are obtained using 1.5 mL of ACN. The decrease of the extraction efficiency at higher volumes of ACN can be related to dilution effect. As mentioned above in all cases,

only 1 mL of the separated phase was used in the DLLME step. It is mentioned that generally 1 mL disperser is used for 5 mL aqueous sample in DLLME procedures. Also, it was verified from the experiments performed using 0.5–2.5 mL ACN as a disperser solvent in DLLME. Therefore, 1.5 mL ACN was selected as the optimum volume of ACN in the further experiments to obtain 1.1 mL separated phase volume.
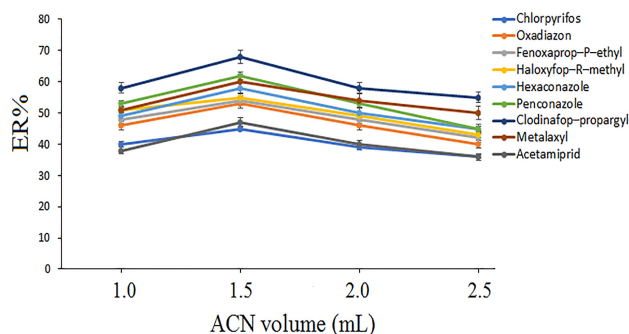

Figure 2. Study of ACN volume. Conditions: HLLE step: sample, 1 mL pesticide–free plasma spiked with 250 μg L⁻¹ of each pesticide; precipitation agent/extraction solvent, ACN; and vortex time, 1 min. DLLME step: aqueous phase, 5 mL HPLC–grade water; without pH adjusting and salt addition; extraction solvent (volume), dichloromethane (120 μL); and centrifugation rate and time, 4000 rpm and 4 min, respectively. The error bars indicate the standard deviations of three repeated determinations.

Optimization of vortex time

In the present work, vortex agitation was utilized to decrease the extraction time. Therefore, vortex agitation time should be optimized. To investigate this parameter, it was studied in the range of 0.5–3.0 min. Considering the obtained results, ERs of the analytes slightly increased till 2.0 min and after that remained constant. Consequently, 2.0 min was chosen for the next studies.

Study of cooling time

In this study, for the first time, liquid nitrogen was used as a coolant in HLLE. It is noted that the other coolants

such as using mixture of salt and ice or freezer needs more time (several minutes to several hours) to perform the phase separation phenomenon. Therefore, liquid nitrogen (–196 °C) was selected as the coolant in the present work to decrease the extraction time. However, when the homogenous solution of ACN–water was placed into the liquid nitrogen for a relatively long time, both of aqueous phase and ACN were frozen. In this case, it was necessary to remain the tube containing the frozen sample at room temperature for several minutes to defreeze the ACN phase. Therefore, to shorten the extraction time, the cooling time (the time of placement of the homogenous solution into liquid nitrogen) was studied. It was observed that when the cooling time was in the range of 10–20 s, phase separation and freezing the aqueous phase were completed. When < 8 s cooling time was used no phase separation was occurred. On the other hand, in cooling times more than 25 s, ACN phase was also frozen. So, 10 s was chosen for the next studies.

Optimization of DLLME parameters
Selection of extraction solvent type
The selection of a suitable extractant is a critical experimental factor in a DLLME procedure. In this study, the extractant should have the following characteristics: (a) higher density than water, (b) low solubility in water, (c) miscibility with ACN, (d) extraction capability for the compounds of interest, and (e) high volatility for easy and fast removal after extraction. Based on these characteristics some organic solvents such as dichloromethane, chloroform, 1,1,1–TCE, and carbon tetrachloride were tested. For this purpose, various volumes of each solvent (70, 120, 65, and 75 μL of 1,1,1–TCE, dichloromethane, carbon tetrachloride, and chloroform, respectively) were used to reach the same volume of the sedimented phase (50 μL) after applying the extraction procedure. As it can be seen in Figure 3, the highest ERs for the analytes are obtained when 1,1,1–TCE is used as the extractant. Therefore, it was selected for the further experiments.
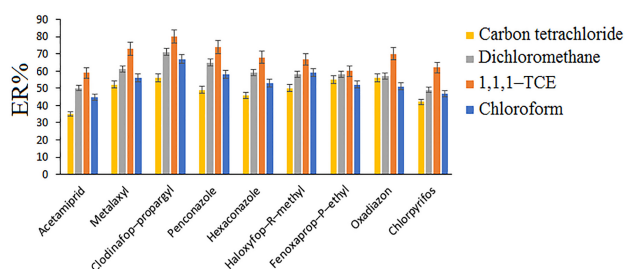

Figure 3. Selection of extraction solvent type in DLLME. Conditions: the same as those used in Fig. 1, except ACN volume and vortex time which were 1.5 mL and 2.0 min, respectively.

Salt addition
Commonly, addition of a salt can influence the ERs of the analytes in different ways (salting–out, salting–in or no effect).[20] In salting–out effect, adding a salt enhances the extraction performance of the analytes by reducing the analytes solubility in the aqueous phase through increasing polarity of the aqueous solution. While, in salting–in effect addition of a salt can increase the viscosity of the aqueous solution which leads to the decreased diffusion coefficients and ERs. To evaluate the effect of this parameter, various concentrations of sodium chloride (0–10%, *w/v*) were tested. Considering the results, salting–in effect was predominant in this study and by enhancing the sodium chloride concentration, the ERs decreased. Therefore, the further studies were done without salt addition.

Study of extraction solvent volume
The extractant volume is a critical parameter that can affect the ERs and detection limits of the method. To evaluate this parameter, the volume of 1,1,1–TCE was varied from 40 to 100 μL. By increasing the volume of 1,1,1–TCE in the mentioned range, volume of the sedimented phase increased from 25 to 85 μL. On the basis of the obtained results, the ERs enhanced with increasing the volume of the extractant till 70 μL and after that remained constant. Thus, 70 μL of 1,1,1–TCE was selected for the next studies.

Optimization of pH
The efficiency of the suggested approach may be affected by varying the pH of aqueous phase because of possible decomposition of the analytes in different pHs. The pH of aqueous solution was investigated from 2 to 12 by adding suitable volumes of 0.1 M HCl or NaOH solution. The obtained outcomes indicated that the ERs of the analytes were pH–independent in the pH range of 4–8. But outside of this interval, the ERs of the analytes decreased remarkably. Since the pH of the deionized water used in this study was 6.8, therefore, there was no need to adjust pH.

Quantitative aspects
To validate the proposed approach, numerous parameters such as limit of detection (LOD), limit of quantification (LOQ), linear range, coefficient of determination (r^2), intra– and inter–day precisions, and ER were studied and the outcomes are summarized in Table 1. Matrix-matched calibration method was used to evaluate the linearity of the method and the results indicated that it was linear in a wide range. The r^2 values were ≥ 0.994. The LODs and LOQs (calculated as the concentrations with signal to noise ratios of 3 and 10, respectively) were in the ranges of 1.4–2.3 and 4.8–7.8 μg L^{-1}, respectively. By evaluating the repeatability of the method using 20 μg L^{-1} standard solutions with respect to each analyte, RSDs%, were computed. The obtained RSDs% were in the ranges of 3–5% and 4–6% for intra– (n=6) and inter–day (n=4) precisions, respectively. The ERs for the analytes were in the range of 59–80%. High ERs, low LODs and LOQs, and good repeatability were achieved using the suggested approach.

Real samples analysis
To investigate the applicability of the suggested approach

Table 1. Quantitative features of the presented method for the selected pesticides.

| Analyte | LOD [a] (μg L^{-1}) | LOQ [b] (μg L^{-1}) | LR [c] (μg L^{-1}) | r^2 [d] | RSD % [e] | | ER ± SD [f] |
					Intra–day (n = 6)	Inter–day (n = 4)	
Acetamiprid	1.7	5.7	5.7–10000	0.994	3.4	4.2	59 ± 4
Metalaxyl	1.4	4.8	4.8–10000	0.995	5.3	6.4	73 ± 2
Clodinafop–propargyl	2.1	6.9	6.9–5000	0.996	3.4	4.8	80 ± 2
Penconazole	2.1	7.0	7.0–10000	0.994	3.4	4.4	74 ± 4
Hexaconazole	2.3	7.6	7.6–10000	0.997	4.9	5.2	68 ± 3
Haloxyfop–R–methyl	1.9	6.4	6.4–10000	0.994	3.4	5.2	67 ± 2
Fenoxaprop–P–ethyl	2.3	7.7	7.7–5000	0.995	4.4	4.9	60 ± 3
Oxadiazon	2.1	6.9	6.9–5000	0.996	4.4	6.2	70 ± 4
Chlorpyrifos	2.3	7.8	7.8–5000	0.995	4.2	4.4	62 ± 3

a) Limit of detection (S/N=3).
b) Limit of quantification (S/N=10).
c) Linear range.
d) Coefficient of determination.
e) Relative standard deviation for intra– and inter–day precisions at a concentration of 20 μg L^{-1} of each analyte.
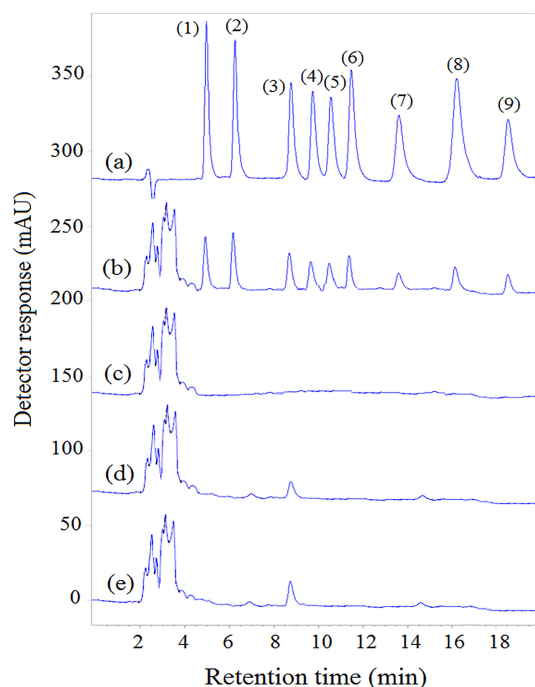f) Extraction recovery ± standard deviation (n=3).

Figure 4. Typical HPLC–DAD chromatograms of: (a) standard solution (5 mg L^{-1} of each analyte in methanol), (b) pesticide–free plasma spiked with 50 μg L^{-1} of each pesticide, (c) pesticide–free (blank) plasma, (d) unspiked plasma sample of farmer 2, and (e) unspiked plasma sample of farmer 5 after performing the proposed method, except chromatogram (a) in which direct injection without preconcentration was used. Detection wavelength was 212 nm. Peaks identification: (1) acetamiprid, (2) metalaxyl, (3) clodinafop–propargyl, (4) penconazole, (5) hexaconazole, (6) haloxyfop–R–methyl, (7) fenoxaprop–P–ethyl, (8) oxadiazon, and (9) chlorpyrifos.

in the analysis of the target pesticides, five plasma samples obtained from the farmers were analyzed under the optimum conditions established above. Considering the outcomes, all of the tested plasma samples were free of the studied pesticides, except two samples. They belonged to the farmers who sprayed clodinafop–propargyl in their farms 2 h before blood sampling. The concentration of clodinafop–propargyl in these samples was 21 ± 2 and 34 ± 1 μg L^{-1} for three determinations. Figure 4 shows HPLC–DAD chromatograms of a directly injected standard solution

(5 mg L^{-1} of each pesticide in methanol), pesticide–free plasma spiked with 50 μg L^{-1} of each pesticide, pesticide–free plasma, and unspiked plasma samples of the above mentioned farmers after applying the introduced method. The added–found method was utilized to evaluate matrix effect in the tested plasma samples. The samples were spiked with the analytes at two concentrations (20 and 50 μg L^{-1} of each analyte) and introduced to the suggested approach. Mean relative recoveries (the recoveries obtained for the analytes in the plasma samples of farmers in comparison with the recoveries obtained in the pesticide–free plasma spiked at the same concentrations) were obtained in the range of 87–102%, which reveal that the matrices of samples have no significant impact on the performance of the suggested method.

Conclusion
In the present work, a two–step procedure based on combination of cold induced–HLLE and DLLME was applied for the extraction and enrichment of nine pesticides in the plasma samples of farmers before their quantitative analysis with HPLC–DAD. In this study, precipitation of plasma proteins and extraction of the analytes were done in one step using ACN which had the role of a disperser solvent in the following DLLME procedure. It led to minimize the use of organic solvents which helped to reduce the risk of approach for human health and environment. In addition, the proposed method was fast using liquid nitrogen for phase separation that decreased the extraction time. The obtained experimental results indicated that the developed procedure provided good repeatability (RSD ≤ 5%), low LODs (1.4–2.3 μg L^{-1}) and LOQs (4.8–7.8 μg L^{-1}), and high ERs (59–80%). These results reveal that the suggested approach can be applied as a low cost and rapid analytical method in the toxicological and risk assessment studies.

Ethical Issues
The volunteers were properly informed about the study and they signed written consent forms.

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