



Combination of solid-phase extraction with dispersive liquid–liquid microextraction followed by GC–MS for determination of pesticide residues from water, milk, honey and fruit juice



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ABSTRACT

In this work, an effective preconcentration method for the extraction and determination of traces of multi-residue pesticides was developed using solid-phase extraction (SPE) coupled with dispersive liquid–liquid microextraction and gas chromatography–mass spectrometry (GC–MS). Variables affecting the performance of both extraction steps such as type and volume of elution and extraction solvents, breakthrough volume, salt addition, extraction time were thoroughly investigated. The proposed method resulted in good linearities ($R^2 > 0.9915$) over the ranges of 1–10,000 ng kg^{−1}, limits of detection (LODs) in the range of 0.5–1.0 ng kg^{−1} at S/N = 3, and precision of RSD% of ≤ 11.8 . Under optimal conditions, the preconcentration factors were obtained in the range of 2362–10,593 for 100 mL sample solutions. Comparison of the proposed method with other ones demonstrated that SPE–DLLME method provides higher extraction efficiency and larger preconcentration factor for determination of pesticides residues. Further, it is simple, inexpensive, highly sensitive, and can be successfully applied to separation, preconcentration and determination of the pesticides (and other noxious materials) in different real food samples.

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1. Introduction

Multi-residue surveillance of pesticides in agricultural products is an ongoing project for regulatory agencies, contract laboratories, and industrial laboratories worldwide. Organic contaminants present in the environment are a result of different sources of pollution from anthropogenic activities. The pesticides, generated by the intensification of agriculture, are regarded as one of the most dangerous contaminants of the environment, despite their numerous merits. They are not only toxic but also are mobile and capable of bioaccumulation. On top of this, they can take part in various physical, chemical and biological processes. Due to these physico-chemical characteristics and their extensive use, many of these

pesticides end-up in surface and groundwater and in agricultural products. They are found nowadays in all surface waters and in a growing number of aquifers. Their presence in water is considered as a potential risk not only for drinking water quality and human health, but also for ecosystems (Dahmardeh, Barghi, Bahramifar, & Esmaili-Sari, 2012; Konstantinou, Hela, & Albanis, 2006; Lozowicka, 2015; Schäfer et al., 2012).

About 26,000 tons of pesticides are distributed to farmers in Iran every year. Since 1994, the government has started a number of programs to reduce the use of pesticides. However, most of these initiatives did not fully incorporate bottom-up participatory approaches and the relevant stakeholders failed to establish sustainable plant management systems at the farm level (Heidari, 2007). Totally 48 pesticides selling shops and 2 plants preservation organizations were questioned. The investigations showed that there were 60 sorts of pesticides in various agriculture and health

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sectors. They included organophosphorus (28.45%), organochlorine (10%), pyrethroids (10%), carbamates (10%), and other derivatives (41.6%). Out of total amount of the used pesticides in a year, 43.65% insecticides, 12.9% acaricides, 17.7% herbicides, 12.9% fungicides, 1.6% molluscicides, and 11.1% rodenticides have been used (Dehghani et al., 2011).

Due to their potential risks to the environment and human health, several commonly occurring pesticides have been included in the priority list of pollutants by the United States environmental protection agency (EPA), WHO, Codex alimentarius and several other organizations. Therefore, there is an increasing demand for reliable and sensitive analytical methods for monitoring and determination of trace levels of these compounds in water, food and environmental matrices. Determination of pesticides in different sample matrices usually carried out after pretreatment step using gas chromatography (GC)–mass spectrometry (MS) (Rodrigues et al., 2011; Wong et al., 2003; Zacharis, Petros, Zachariadis, & Zotos, 2012), GC-electron capture and ion trap mass spectrometric detectors (Jeong, Kwak, & Ahn, 2012; Yazdanfar, Yamini, & Ghambarian, 2014), GC-nitrogen-phosphorus detector (Salemi, Rasoolzadeh, Mohebbi Nejad, & Vosough, 2013), GC-flame photometric detector (Khalili-Zanjani, Yamini, Yazdanfar, & Shariati, 2008; Samadi, Sereshti, & Assadi, 2012), GC-flame ionization detector (Naeeni, Yamini, & Rezaee, 2011), and high performance liquid chromatography (HPLC) with different detectors, such as MS/MS spectrometry (Barganska, Slebiada, & Namiesnik, 2013; Tian, 2011), diode array detector (Melo, Aguiar, Mansilha, Pinhod, & Ferreira, 2012). The use of ultra-performance liquid chromatography in detection of pesticides has also been reported (Galán-Cano, Lucena, Cárdenas, & Valcárcel, 2013).

Before analysis, due to the complexity of some sample matrices, incompatibility of sample medium with the instrument, and the low concentration of the analytes in real samples, a preliminary sample preconcentration and/or separation technique is required. Thus, different preconcentration methods such as solid-phase extraction (SPE) (Cavaliere, Monteleone, Naccarato, Sindona, & Tagarelli, 2012; Wong et al., 2003), solid-phase microextraction (SPME) (Melo, Aguiar, Mansilha, Pinhod, & Ferreira, 2012), liquid-phase microextraction (LPME) (Khalili-Zanjani, Yamini, Yazdanfar, & Shariati, 2008), single drop microextraction (SDME) (Tsiropoulos & Amvrazi, 2011), headspace solid-phase microextraction (HSPME) (Rodrigues et al., 2011), dispersive liquid–liquid microextraction (DLLME) (Zacharis, Petros, Zachariadis, & Zotos, 2012), homogeneous liquid–liquid microextraction (HLLME) (Yazdanfar, Yamini, & Ghambarian, 2014), ultrasonic assisted headspace single drop microextraction (USA-HSDME) (Salemi, Rasoolzadeh, Mohebbi Nejad, & Vosough, 2013), vortex-assisted liquid–liquid microextraction (VALLME) (Jia et al., 2010), ultrasound-assisted solvent extraction followed by dispersive liquid–liquid microextraction (Bidari, Ganjali, Norouzi, Milani Hosseini, & Assadi, 2011), combination of supercritical fluid extraction with dispersive liquid–liquid microextraction (Naeeni, Yamini, & Rezaee, 2011), microwave-assisted extraction solid-phase extraction (Fang, Lau, Law, & Li, 2012), and SPE in combination with DLLME (Cristina et al., 2011) have been used for preparation of water samples containing pesticides. SPE–DLLME is an efficient hyphenated technique that offers the advantages of both methods such as simplicity, low solvent usage and exposure, low disposal costs and extraction time, with high recovery and enrichment factor.

We have recently applied the SPE–DLLME method to the preconcentration and determination of traces of some inorganic (Fattahi, Shamsipur, Assadi, Sadeghi, & Sharafi, 2014) and organic compounds in different real samples (Hashemi, Shamsipur, & Fattahi, 2015). In the present work, we also applied SPE–DLLME to parts per trillion (ppt) level determinations of pesticides in water and food samples. Here, 19 of the most common agricultural

pesticides used in Iran were selected. Preconcentration was performed in two stages, SPE followed by DLLME. The quantitative analyses were performed by the use of GC–MS, and the effects of various experimental parameters on extraction efficiency of pesticide residues from water and food samples were considered and optimized.

2. Experimental

2.1. Reagents and materials

All pesticides (Dichlorovos, Carbaryl, Diazinon, Primicarb, Metaxyl, Fenitrothion, Malathion, Aldrin, Profenophos, Ethion, DDT, Bromopropylate, Fenpropathrin, Tetradifon, Phosalone, Permethrin, Dimethoxybenzamide, Deltamethrin, and Fenvalarate) were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol, ethanol, THF, acetone, carbon tetrachloride, chloroform, carbon disulfide, chlorobenzene, sodium chloride and other solvents were of the highest purity available from Merck (Darmstadt, Germany). The water used for preparation of aqueous solutions was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Proper amount of each pesticide was dissolved in acetone or methanol to obtain a stock standard solution with a concentration of 5000 mg L⁻¹. A fresh standard solution containing pesticides with concentration of 100 mg L⁻¹ was prepared in acetone every week and stored at 4 °C.

2.2. Instrumentation

Determination of the target analytes was performed using an Agilent 7890A gas chromatograph equipped with a 5975C mass selective detector (MSD, Agilent Technologies) and a Chrompack CP-Sil 8 CB fused-silica capillary column (50 m × 0.25 mm i.d. and 0.25 μm film thickness). Helium (purity 99.9999%) was employed as the carrier gas at a flow rate of 1 mL min⁻¹. During the whole analysis, the injector was operated in the splitless mode with an injector temperature of 280 °C. The oven temperature was initially set at 50 °C (2 min hold), followed by a temperature ramp of 10 °C min⁻¹ to 200 °C, and held for 5 min, then increased to 280 °C at a rate of 5 °C min⁻¹ (10 min hold), for a total run time of 55 min. The MSD was operated in electron impact (EI) mode at 70 eV. The inlet, MSD transfer line, MSD source, and quadrupole temperatures were 280, 280, 230, and 150 °C, respectively. The sample extracts, standards, and blanks were injected (1 μL) into the GC. The MSD system was routinely programmed in selective ion monitoring (SIM) according to the conditions selected for any pesticide, using two ions (as designated in Table 1). Confirmation of the pesticides was established by the retention time and the presence of the target ions. The target ion abundances were determined by injection of individual pesticide standards under the same chromatographic conditions, but utilizing full-scan conditions with the mass/charge scan ranging from 40 to 550 *m/z*.

Our preliminary experiments showed that the impurities of one of the chlorobenzene, as extraction solvents, were eluted around 15.3 min from the column, which is located near the analyte peaks and its peak area depends only on the injection volume. Thus, in order to increase the reproducibility of the detection system, this impurity was used as the internal standard, as also reported before (Zanjani, Yamini, Shariati, & Jönsson, 2007). Consequently, the analytical signal was taken as the ratio of analyte peak area to that of the internal standard.

2.3. Pretreatment of real samples

The original water samples collected were stored in pre-cleaned polyethylene bottles in a fridge at about 4 °C under darkness

Table 1

Analytical characteristics of SPE–DLLME/GC–MS for determination of multi-residue pesticides.

No.	Pesticide	RT	M/Z	PF	LOD (ng kg ⁻¹)	LR (ng kg ⁻¹)	Intra-day precision (RSD%, n = 5)		Inter-day precision (RSD%, n = 5)		R ²
							500 ng kg ⁻¹	5000 ng kg ⁻¹	500 ng kg ⁻¹	5000 ng kg ⁻¹	
1	Dichlorovos	14.73	109, 185	6328	0.5	5–5000	7.51	10.53	10.83	8.07	0.9967
2	Carbaryl	22.52	115, 144	3929	1	10–10,000	9.20	8.68	6.79	6.87	0.9949
3	Diazinone	23.89	137, 179	7862	0.5	100–10,000	0.76	0.41	1.89	1.20	0.9996
4	Primicarb	25.07	72, 166	3003	1	5–10,000	9.79	10.25	8.57	9.96	0.9985
5	Metalaxyl	26.72	45, 206	2362	1	10–10,000	10.73	11.75	11.09	10.61	0.9971
6	Fenitrothion	27.36	125, 277	10,459	0.5	10–10,000	3.81	1.69	4.39	2.51	0.9991
7	Malathion	27.72	125, 173	10,593	0.5	5–10,000	4.72	4.26	6.09	6.62	0.9995
8	Aldrin	28.23	66, 263	3007	1	5–10,000	5.65	5.47	3.25	5.57	0.9977
9	Profenophos	32.20	208, 337	7163	0.5	1–10,000	3.67	1.65	5.61	3.63	0.9976
10	Ethion	34.20	153, 231	4532	0.5	1–10,000	4.18	2.98	4.23	5.47	0.9994
11	DDT	35.57	165, 235	2704	1	5–5000	7.54	6.60	6.80	4.72	0.9956
12	Bromopropylate	37.55	183, 341	10,593	0.5	5–10,000	4.72	4.26	6.09	6.62	0.9995
13	Fenpropathrin	37.82	97, 181	3539	1	5–10,000	10.43	11.62	11.17	10.28	0.9950
14	Tetradifon	38.65	111, 159	5211	0.5	1–10,000	6.72	7.84	7.46	8.42	0.9951
15	Phosalone	39.00	121, 182	7847	1	5–5000	3.25	1.95	4.85	2.21	0.9946
16	Permethrin	41.49	163, 183	3595	1	500–5000	3.59	4.71	5.08	4.90	0.9941
17	Dimethoxybenzamide	41.88	165, 181	3595	0.5	1–5000	8.64	8.07	9.00	8.15	0.9915
18	Deltamethrin	47.98	181, 253	4675	1	1–5000	10.25	11.34	12.15	11.00	0.9915
19	Fenvalarate	48.81	125, 419	4502	1	5–5000	10.31	9.27	11.25	10.21	0.9928

RT, retention times of GC–MS chromatogram; M/Z, selected ions for target pesticides used as quantifier and qualifier respectively; RSD at concentrations of 500 and 5000 ng kg⁻¹ of each pesticides in aqueous sample solution, intra and inter day; R², square of correlation coefficient; LOD, limit of detection for S/N = 3; RSD, relative standard deviation.

condition. The samples were filtered through a 0.45 mm pore size cellulose acetate membrane filter prior to extraction. For preparation of honey samples, 20 g honey was transferred quantitatively to a 100 mL volumetric flask, and the flask filled to the mark with distilled water and, subsequently, the solution was filtered through a 0.45 mm pore size cellulose acetate membrane filter prior to extraction. Milk and fruit juices were purchased from the retail market. In order to remove the potential interfering substances, firstly, 20 mL of milk or orange juice samples was diluted with distilled water in a 100 mL Erlenmeyer flask. Then, 1.0 mL of Carrez solution I (containing potassium hexacyanoferrate(II) trihydrate, 15% w/v in water) was added and mixed carefully. Then, 1.0 mL of Carrez solution II (containing zinc sulfate heptahydrate, 30% w/v in water) was added and mixed again. The contents of the Erlenmeyer flasks were transferred quantitatively to 100 mL volumetric flasks, and were filled to the mark with distilled water. Subsequently, the solution was filtered through a fluted filter or centrifuged to separate the insoluble compounds prior to the SPE–DLLME procedure.

2.4. Extraction procedures

2.4.1. Solid-phase extraction of pesticides

SPE cartridge used for the extraction of pesticides was 500 mg C₁₈ sorbent (6-mL syringe barrel, Varian, Harbor City, CA, USA). The sorbent was conditioned with 3 mL of methanol and ultra-pure water, respectively. A 100 mL volumetric flask was spiked with a given volume of a 20 µg L⁻¹ mixture of pesticides and the flask was filled up to the mark with ultra-pure water. The sample was loaded at a flow rate of about 15 mL min⁻¹ with the aid of a vacuum pump (Rotavac, Heidolph, Germany). The C₁₈ cartridges were rinsed with 3 mL of water to remove the matrix interferences. After drying the solid phase by passing air through it, the pesticides were eluted with 1.5 mL methanol and were collected in a capped glass test tube.

2.4.2. DLLME protocol

An aliquot of 5 mL of 1 mol L⁻¹ NaCl solution was transferred to a 10 mL screw capped glass tube with conical bottom. A mixture of 1.5 mL eluted solution from SPE stage (as disperser solvent) and

20 µL chlorobenzene (as extraction solvent) was rapidly injected into the solution in the 10 mL screw capped glass test tube and then the mixture was gently shaken by hand for 1 min. A cloudy solution, resulting from the dispersion of the fine chlorobenzene droplets in the aqueous solution, was formed in the test tube. In this step, the pesticides were extracted into the fine chlorobenzene micro droplets. The mixture was centrifuged for 6 min at 2500 rpm for phase separation, so that the dispersed fine droplets of the extraction phase was settled to the bottom of the conical test tube (10.0 ± 0.3 µL). The sediment phase was completely transferred into micro vial sample cup using a 10 µL syringe (gastight, Hamilton, Reno, NV, USA) and 1 µL of this sediment phase using for analysis by GC–MS/SIM.

3. Results and discussion

In this research, for the first time, a SPE–DLLME method combined with GC–MS was developed for preconcentration of ultra-traces of pesticide residues. Mixture of 19 pesticides was chosen as an example to study the applicability of this combination. To attain a high preconcentration factor, the influence of different parameters affecting the extraction performances of SPE and DLLME, such as flow rates, breakthrough volume, type and volume of disperser and extraction solvents, extraction time, and salt addition were optimized. The study and the optimization of the above mentioned variables were performed using one variable at a time method. The chromatographic peak area was the parameter used to evaluate the influence of the factors on the extraction efficiency of pesticides by the SPE–DLLME.

3.1. Optimization of SPE parameters

3.1.1. Effect of the flow rate of the sample solution

The flow rate of the sample solution must be low enough to perform an effective retention of the pesticides. On the other hand, it must be high enough not to waste the time. The effect of the flow rate of aqueous sample solution with concentration of 20 ng g⁻¹ of pesticides was examined from 5 to 30 mL min⁻¹ (Samadi, Sereshti, & Assadi, 2012; Shamsipur, Fattahi, Assadi, Sadeghi, & Sharafi,

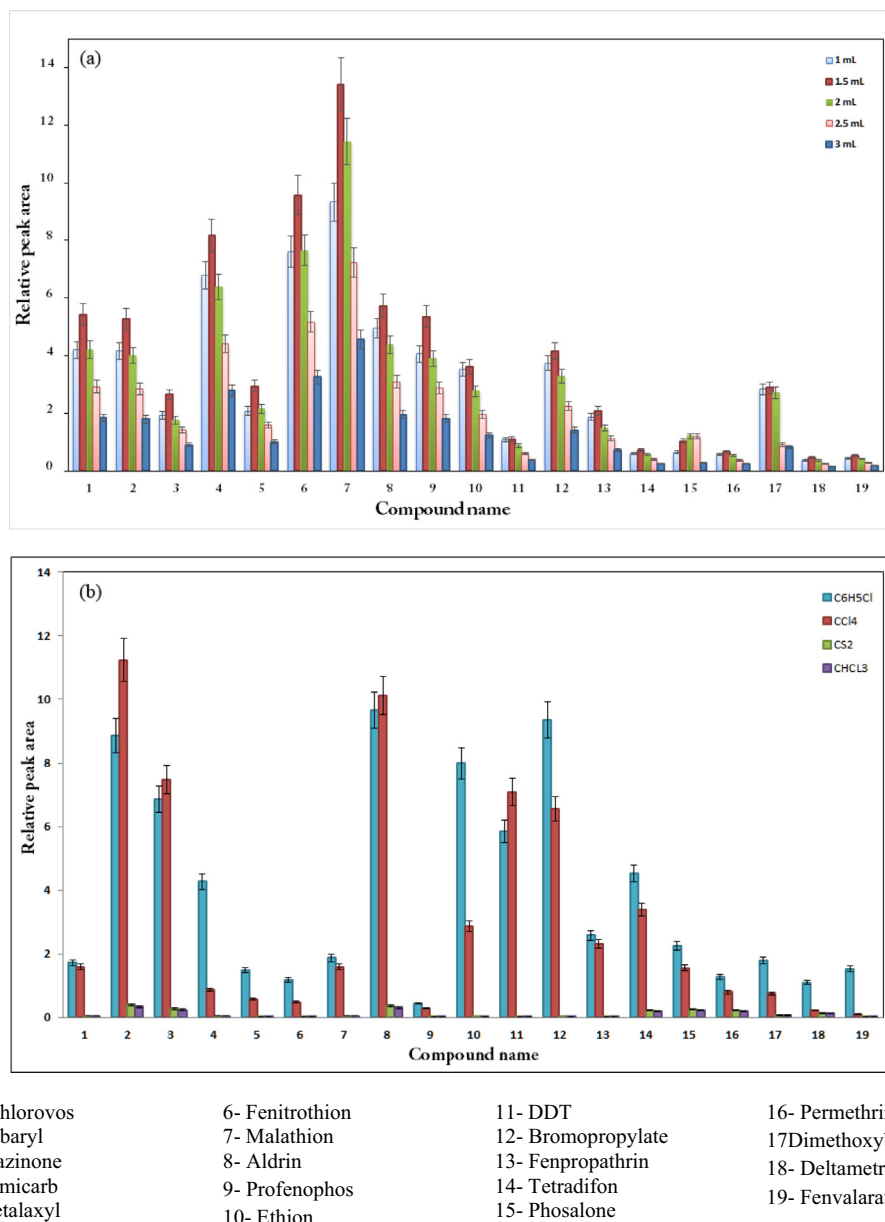


Fig. 1. Effects of elution solvent volume (a) and extraction solvent type (b) on the extraction efficiency of pesticide residues. Conditions: extraction solvent volume, 20 μ L; eluent type, methanol; water sample volume, 100 mL; stirring rate, 2500 rpm; NaCl concentration in DLLME solutions 1 mol L⁻¹.

2014). It was found that the flow rate up to 15 mL min⁻¹ resulted in the highest preconcentration factor and preconcentration factor decreased at higher speeds (Fig. S1, Supplementary data). Thus, 15 mL min⁻¹ was selected for optimal condition.

3.1.2. Effect of pH and sample volume

According to the structure of pesticides compounds studied and the results reported in previous works (Samadi, Sereshti, & Assadi, 2012), the pH of sample solution found to have no significant effect on the extraction recovery of target pesticide residues. Thus, no pH adjustment was carried out in further experiments.

In the analysis of ultra-trace of pesticide residues, the sample volume is one of the critical parameters influencing the preconcentration factor. In order to achieve a high preconcentration factor for sample with very low analyte concentration, a large volume of samples solution is required. Therefore, the effect of sample volume on the retention of pesticides was investigated. For this

purpose, different sample volumes (20, 50, 100, 200, and 300 mL) of the aqueous sample solution containing 2 μ g of pesticides were preconcentrated in the C18 cartridge (Fig. S2, Supplementary data). Considering the analytical time and trace level of pesticides in real samples, 100 mL was used as the optimized breakthrough volume.

3.1.3. Effect of the elution solvent type

In SPE–DLLME procedure, the elution solvent of SPE must also play the role of a disperser solvent at the DLLME stage. For this purpose, acetone, THF, acetonitrile, ethanol, and methanol, displaying this ability, were selected as the elution solvent. The C18 cartridge was eluted using 1.5 mL of each elution solvent. The results indicated that the preconcentration factor by using methanol is more effective than the other potential elution solvents. Therefore, methanol was selected as the elution solvent in further experiments.

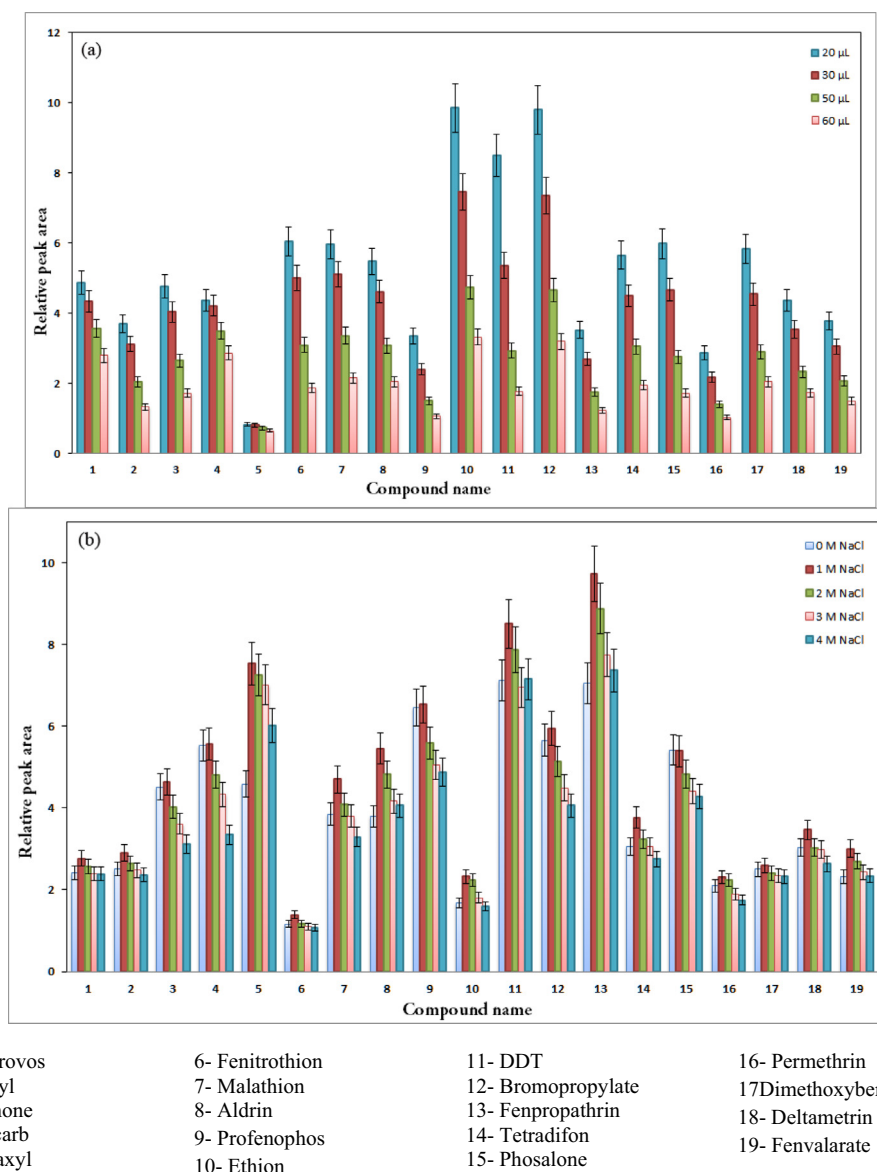


Fig. 2. Effects of extraction solvent volume (a) and salt addition (b) on the extraction efficiency of pesticide residues. Conditions: extraction solvent, chlorobenzene; eluent (methanol) volume, 1.5 mL; water sample volume, 100 mL; stirring rate, 2500 rpm.

3.1.4. Effect of the elution solvent volume

For obtaining optimized volume of elution solvent, various experiments were performed by using different volumes of methanol (0.5, 1.0, 1.5, 2.0, and 2.5 mL). Fig. 1a shows the peak area of the resulting signals versus volume of methanol as elution solvent. According to the results show in Fig. 1a, the methanol volumes lower than 1.50 mL cannot elute the cartridge and the preconcentration factor decreases. Also, at methanol volumes of higher than 1.50 mL, the preconcentration factor decreases because of the increasing solubility of extraction solvent in water. Therefore, a volume of 1.50 mL was chosen as the optimum volume of the elution solvent.

3.2. Optimization of DLLME parameters

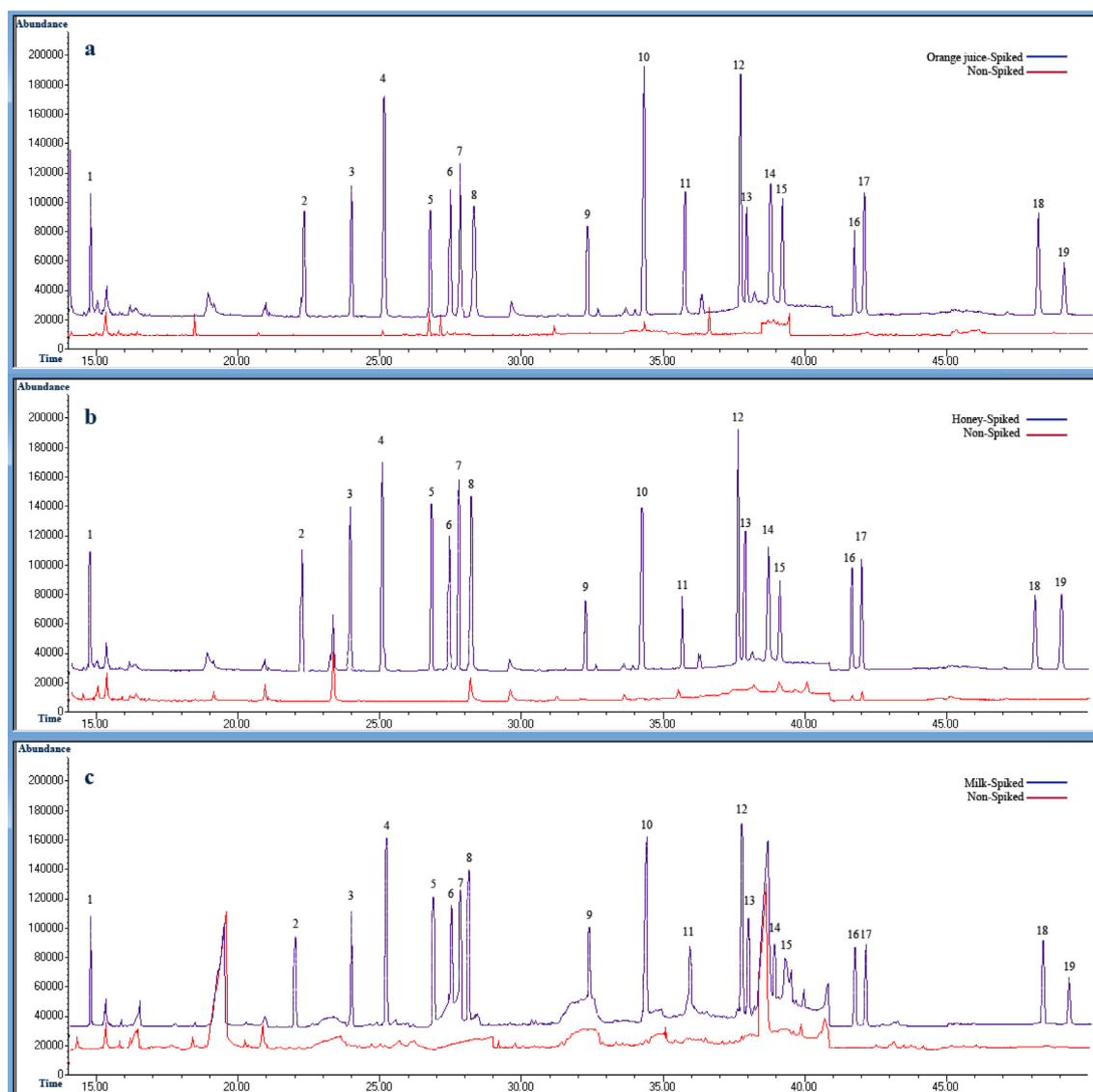
3.2.1. Effect of the type of extraction solvent

The extraction solvent must possess given properties, including higher density than water, high extraction capability of the analytes, and low solubility in water. To investigate the effect of

extraction solvent, chloroform, carbon tetrachloride, chlorobenzene, and carbon disulfide were tested using 1.50 mL methanol, as disperser solvent, containing different volumes of the extraction solvents to achieve a sediment phase volume of 10.0 ± 0.3 µL. The results illustrated in Fig. 1b clearly indicate that, in the case of most pesticides, the preconcentration factor obtained by the use of chlorobenzene is higher than that of other extraction solvents. Therefore, chlorobenzene was selected as the extraction solvent in further experiments.

3.2.2. Effect of the extraction solvent volume

In order to examine the effect of the extraction solvent volume, additional experiments were performed by using 1.50 mL methanol, as disperser solvent, containing different volumes of the extraction solvent chlorobenzene (10.0, 20.0, 30.0, 40.0, 50.0, and 60.0 µL), and the recommended procedure was followed (Fig. 2a). It was found that the increasing volume of chlorobenzene from 20.0 to 60.0 µL, resulted in an increase of the sedimented phase, approximately from 10 to 50 µL. As the volume of the sedimented



1- Dichlorvos
2- Carbaryl
3- Diazinone
4- Primicarb
5- Metalaxyl

6- Fenitrothion
7- Malathion
8- Aldrin
9- Profenophos
10- Ethion

11- DDT
12- Bromopropylate
13- Fenpropathrin
14- Tetradifon
15- Phosalone

16- Permethrin
17- Dimethoxybenzamide
18- Deltametrin
19- Fenvalarate

Fig. 3. Chromatograms of blank (red) and spiked (blue) orange juice (a), blank (red) and spiked (blue) honey (b) and blank (red) and spiked (blue) milk (c), with 5000 ng kg^{-1} of each pesticides, obtained by SPE-DLLME-GC-MS method. Extraction conditions: extraction solvent, chlorobenzene, $20 \mu\text{L}$; eluent, methanol, 1.5 mL ; water sample, 100 mL ; stirring rate, 2500 rpm with addition of 1 mol L^{-1} of NaCl into DLLME solutions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phase increases with increasing volume of chlorobenzene, the pre-concentration factor decreases. Subsequently, volumes of lower than $20 \mu\text{L}$ found to be inadequate to result in enough volume of sediment. According to the results thus obtained, $20.0 \mu\text{L}$ of chlorobenzene was selected as the optimum extraction solvent volume.

3.2.3. Salt effect

In DLLME process, phase separation phenomenon and the volume of chlorobenzene sedimented are dependent on NaCl concentration. In order to investigate the optimum amount of NaCl in the quantitative DLLME of pesticides, the experiments were carried out

by changing the concentration of NaCl in the sample solution in the range of $0\text{--}4 \text{ mol L}^{-1}$. As shown in Fig. 2b, the increase in ionic strength of the aqueous solution promotes the transport of analytes into the organic phase, so the pre-concentration factor increases with the increase in NaCl concentration up to 1 mol L^{-1} . At higher concentrations of NaCl, a decrease in signal is occurred most possibly due to increase in viscosity and/or a change of the physical properties of the Nernst diffusion film, which decrease the mass transfer of the analytes to the organic solvent; consequently, the pre-concentration factor of the analytes decrease. Therefore, 1 mol L^{-1} of NaCl was added into the DLLME solutions in the subsequent studies.

Table 2

Comparison of SPE–DLLME/GC–MS with other methods for determination of pesticides residue.

Methods	LOD ^a (ng g ⁻¹)	LR ^b (ng kg ⁻¹)	RSD ^c %	Samples	Ref.
SPE–GC–MS	10–5000	1000–2500	–	Wine	Wong et al. (2003)
SPME–HPLC–DAD	280	>800,000	7.7	Lettuce	Melo, Aguiar, Mansilha, Pinhod, and Ferreira (2012)
SPME–GC–MS	40–1700	50–10,000	5.4–14.6	Water	Cavaliere, Monteleone, Naccarato, Sindona, and Tagarelli (2012)
LPME–GC–FPD	10–40	10–100,000	3.5–8.9	Water	Khalili-Zanjani, Yamini, Yazdanfar, and Shariati (2008)
SDME–GC–MS	30–10,000	1250–500,000	4–15	Honey	Tsiropoulos and Amvrazi (2011)
DLLME–GC–ECD	20–150	100–20,000	20	Honey	Zacharis, Petros, Zachariadis, and Zotos (2012)
HLLE–GC–ECD	1–30	10–100,000	<8.6	Water & Fruit	Yazdanfar, Yamini, and Ghambarian (2014)
SPE–DLLME–GC–MS	0.5–1	1–10,000	0.1–11.75	Water, Fruit, Honey & Milk	Present method

^a LOD, limit of detection.^b LR, linear range.^c RSD, relative standard deviation.**Table 3a**Relative recoveries and standard deviations of pesticides with and without spiked (500 and 5000 ng kg⁻¹) tap water & agricultural waste water.

Pesticide	Agricultural waste water			Tap water		
	Detected \pm SD ng g ⁻¹	%Recovery (SD) of 500 ng kg ⁻¹ added	%Recovery (SD) of 5000 ng kg ⁻¹ added	Detected \pm SD ng g ⁻¹	%Recovery (SD) of 500 ng kg ⁻¹ added	%Recovery (SD) of 5000 ng kg ⁻¹ added
Dichlorovos	0.77 \pm 0.06	86.5(11.4)	99.3(1.53)	ND	102(7.33)	90.4(13.2)
Carbaryl	ND	90.4(13.2)	98.5(8.68)	ND	81(0.14)	82.7(10.6)
Diazinone	0.56 \pm 0.01	82.7(0.6)	99.4(2.86)	ND	88(3.89)	105.0(1.25)
Primicarb	ND	85.0(4.9)	105(10.25)	ND	78.3(3.89)	96.4(2.98)
Metaxyl	1.83 \pm 0.08	95.9(10.5)	99.8(11.75)	ND	100(3.88)	99.8(11.75)
Fenitrothion	1.03 \pm 0.1	79.4(9.2)	100(1.69)	ND	86(3.11)	103.7(4.26)
Malathion	0.77 \pm 0.01	89.9(0.8)	103.7(4.26)	ND	98(6.08)	104.6(7.4)
Aldrin	ND	93.8(10.8)	98.5(5.47)	ND	84.0(15.9)	97.9(1.65)
Profenophos	ND	80.7(10.5)	97.9(1.65)	ND	85.5(10.6)	105(9.85)
Ethion	ND	86.5(8.9)	96.4(2.98)	ND	78.1(9.9)	99.1(6.60)
DDT	ND	86.7(11.0)	99.1(6.60)	ND	93(8.17)	98.0(11.62)
Bromopropylate	ND	94.0(3.3)	104.6(6.77)	ND	100(9.86)	96.8(7.84)
Fenpropathrin	ND	89.2(4.5)	98.0(11.62)	ND	100(1.17)	98.5(5.47)
Tetradifon	5.47 \pm 0.2	78.5(10.9)	96.8(7.84)	ND	98.0(11.62)	94(3.3)
Phosalone	ND	91.6(11.4)	98.5(1.95)	ND	96.8(7.84)	99(1.57)
Permethrin	ND	87.0(2.6)	102(4.71)	ND	86.3(9.1)	102(4.71)
Dimethoxybenzamide	ND	86.5(1.4)	96.3(8.07)	ND	86.1(6.3)	91.6(11.4)
Deltametrin	ND	90.4(3.2)	98.5(11.34)	ND	83.9(11.4)	87.0(2.6)
Fenvalarate	ND	82.7(9.6)	98.4(9.27)	ND	88.4(12.7)	86.5(1.4)

SD, standard deviation ($n = 3$). ND, not detected or <LOD

3.2.4. Effect of extraction time and centrifugation speed

A main characteristic of the DLLME is the formation of micro droplets of the extraction solvent that is dispersed in the aqueous phase. Thus, the large surface area of contact between both phase results in fast mass transfer process, which provides a fast extraction process. In this sense, the performance of the DLLME was examined at time intervals between 1 and 20 min. As expected, the extraction time was found to have a negligible influence on the peak areas of the analytes and, therefore, a practical time of 1 min was selected. Finally, the effect of centrifugation speed on the extractability of the pesticides was examined in the range of 2000–4000 rpm. The averages of peak areas were slightly improved up to 2500 rpm and being unaffected at higher rates.

3.3. Quantitative analysis

Analytical characteristics of the optimized method, including linear range, limit of detection (LOD), reproducibility, and preconcentration factor are listed in Table 1. As seen, the linearity was observed over the range of 1.0–10,000 ng kg⁻¹ with the coefficient of determination (R^2) in the range of 0.9915–0.9996. The LODs, calculated as the concentration equivalent to three times of standard deviation of the blank divided by the slope of calibration curve, were in the range of 0.5–1 ng kg⁻¹ for different pesticides. The intra-day precision and inter-day precision, at 500 and 5000 ng kg⁻¹ levels, were studied by calculation of RSD% of five replicate extractions and the resulting values are added to Table 1.

As seen, all resulting RSD% values were in an acceptable range of 0.41–11.62. The preconcentration factor (PF), calculated as the ratio of the final concentration of analyte in the sedimented phase and its concentration in the initial solution. The preconcentration factors thus obtained were in the range of 2362–10,593 for a 100 mL water sample. The broad linear dynamic range combined with the low detection limit suggested a high potential for monitoring pesticide residues in various real samples by applying the proposed SPE–DLLME/GC–MSD method.

3.4. Comparison of SPE–DLLME with other methods

A comparison of the main analytical characteristics of the proposed method with other previously studied techniques for determination of pesticide residues is summarized in Table 2. The obtained LODs and LDRs in this study are considerably lower than that of the most of the other methods. RSDs are either comparable or even better than those reported in other studies. It can be concluded that SPE–DLLME/GC–MS is a sensitive method that can be used for the preconcentration and determination of pesticide residues from water and food samples.

3.5. Analysis of real samples

To evaluate the accuracy and reliability of the proposed method (SPE–DLLME) for extraction and preconcentration of the pesticides from real samples, and to investigate the matrix effects on the

Table 3b
Relative recoveries and standard deviations of pesticides with and without spiked (500 and 5000 ng kg⁻¹) honey, milk and orange juice.

Pesticide	Honey			Milk			Orange juice		
	Detected ± SD ng g ⁻¹	%Recovery (SD) of 500 ng kg ⁻¹ added	%Recovery (SD) of 5000 ng kg ⁻¹ added	Detected ± SD ng g ⁻¹	%Recovery (SD) of 500 ng kg ⁻¹ added	%Recovery (SD) of 5000 ng kg ⁻¹ added	Detected ± SD ng g ⁻¹	%Recovery (SD) of 500 ng kg ⁻¹ added	%Recovery (SD) of 5000 ng kg ⁻¹ added
Dichlorovos	ND	99(1.29)	99.3(1.53)	ND	84.5(9.6)	85.5(10.6)	ND	100(9.86)	80.7(10.5)
Carbaryl	ND	81(0.9)	98.5(8.68)	ND	81(0.14)	82.7(8.1)	ND	98.0(3.06)	86.5(1.4)
Diazinone	ND	78(10.7)	99.4(2.86)	ND	88(3.89)	77.5(3.6)	ND	98.0(11.62)	98.8(0.67)
Primicarb	ND	100(3.3)	98.0(6.5)	ND	66.3(3.89)	96.4(2.98)	0.17 ± 0.1	95.8(10.75)	98.0(11.62)
Metalaxyl	ND	86(8.7)	94.8(6.4)	ND	99.1(6.44)	80.7(11.8)	1.29 ± 0.1	86.5(11.4)	72(1.95)
Fenitrothion	ND	79.4(9.2)	100(1.69)	ND	86(3.11)	82.5(2.3)	ND	90.4(13.2)	98.5(5.47)
Malathion	ND	89.9(0.8)	103.7(4.26)	ND	98(6.08)	84.0(5.9)	ND	82.7(10.6)	89.8(0.88)
Aldrin	1.08 ± 0.1	93.8(10.8)	98.5(5.47)	ND	89.1(6.60)	97.9(1.65)	ND	85.0(4.9)	86.1(6.3)
Profenophos	ND	85.5(10.2)	97.9(1.65)	ND	85.5(10.6)	105(9.85)	ND	70(5.5)	99.1(6.60)
Ethion	ND	86.5(8.9)	96.4(2.98)	ND	78.1(9.9)	99.7(6.60)	0.43 ± 0.04	104.6(7.4)	106(3.08)
DDT	ND	86.7(11.0)	86.1(6.3)	ND	93(8.17)	99(2.09)	ND	100(1.85)	102(7.33)
Bromopropylate	ND	94.0(3.3)	99.1(6.60)	ND	76(11.67)	71(4.66)	ND	105(10.25)	100(1.17)
Fenpropathrin	ND	89.2(4.5)	98.0(11.62)	ND	66(4.32)	78(2.9)	ND	99.8(11.75)	73(9.9)
Tetradifon	ND	83.5(1.6)	96.8(7.84)	ND	78(1.6)	94(3.3)	ND	99(1.57)	104.6(6.77)
Phosalone	0.77 ± 0.06	91.6(11.4)	87.5(10.6)	ND	96.8(7.84)	76(4.4)	ND	102(3.88)	98(0.17)
Permethrin	0.29 ± 0.01	87.0(2.6)	102(4.71)	ND	86.3(9.1)	102(4.71)	ND	96.8(7.84)	78.5(10.9)
Dimethoxybenzamide	0.63 ± 0.01	85.5(0.6)	96.3(8.07)	ND	98.0(1.62)	67(11.3)	ND	105(1.12)	100(9.07)
Deltamethrin	ND	90.4(3.2)	79.5(3.6)	ND	83.9(11.4)	87.0(2.6)	ND	91.6(11.4)	100(3.88)
Fenvalerate	ND	82.7(9.6)	98.4(9.27)	ND	88.4(12.7)	86.5(1.4)	ND	90.4(13.2)	93(8.87)

results obtained by the method, two real water samples (i.e., tap water and agricultural waste water) and three food samples (i.e., milk, honey, and fruit juice) were selected, and subjected to the SPE–DLLME–GC–MS analysis.

3.5.1. Analysis of water samples

In order to investigate the matrix effect on the efficiency of method, two aqueous samples were collected from tap water and agricultural waste water from Tehran (Iran) and were prepared based on procedures described in Section 2.3. Then, the target analytes were extracted under the optimal conditions. The blank analysis showed that tap water was free from pesticides contamination, while six pesticides were detected in the agricultural waste water (Table 3a). To assess matrix effects, the water samples were spiked with the pesticides standard solutions at different concentration levels (500 and 5000 ng kg⁻¹). The relative recovery of the pesticides from tap water and agricultural waste water were in the range of 78.1–105% and 78.5–105%, respectively (Table 3a). Therefore, the results indicated that the matrices of the analyzed real water samples possess ignorable effect on the performance of the method.

3.5.2. Extraction of pesticides from honey

Honey sample was prepared from a Beehive near a garden sprayed by pesticides. The preparation of sample was carried out by the procedures described in Section 2.3. Then, the target analytes were extracted under the optimal conditions. As it is seen from Fig. 3b and Table 3b, trace amounts of four pesticides aldrin (8, 1.08 ± 0.1 ng g⁻¹), phosalone (15, 0.77 ± 0.06 ng g⁻¹), permethrin (16, 0.29 ± 0.01 ng g⁻¹) and dimethylbenzamide (17, 0.63 ± 0.01 ng g⁻¹) were detected in blank honey sample. To assess the matrix effects, the honey sample was spiked with two different concentration levels (500 and 5000 ng kg⁻¹) of pesticides' standards. The relative recoveries of analytes were in the range of 78–100% for 500 ng kg⁻¹ and 79.5–103.7% for 5000 ng kg⁻¹, as shown in Table 3b. It is obvious that this method is applicable to honey samples.

3.5.3. Extraction of pesticides from milk and orange juice samples

Milk and fruit juices were purchased from the retail market. The samples were prepared based on procedures described in Section 2.3. Then, the target analytes were extracted under the optimal conditions. The blank analysis showed that milk was free from pesticides contamination (Fig. 3c), while traces of three pesticides primicarb (4, 0.17 ± 0.1 ng g⁻¹), metalaxyl (5, 1.29 ± 0.1 ng g⁻¹) and ethion (10, 0.43 ± 0.04 ng g⁻¹) were detected in orange juice (Fig. 3a and Table 3b). To assess matrix effects, the prepared samples spiked with two different concentration levels (500 and 5000 ng kg⁻¹) of pesticides' standards. The relative recoveries of analytes were in the range of 66–99.1% for 500 ng kg⁻¹ and 67–105% for 5000 ng kg⁻¹ in milk and 70–105% for 500 ng kg⁻¹ and 69.8–106% for 5000 ng kg⁻¹ in orange juice, as shown in Table 3b. As is obvious, the proposed method is applicable to milk and orange juice samples with negligible matrix effects.

4. Conclusions

A sensitive and reliable SPE–DLLME procedure and coupled with GC–MS/SIM was developed and optimized for the quantitative determination of trace-levels of 19 pesticides in water, honey, milk and orange juice. The method provides good precisions, wide linear ranges, and very low limits of detection (ng kg⁻¹) for the analysis of such samples. The combination of SPE–DLLME is not only an excellent sample clean-up but also results in obtaining high preconcentration factors, that can be used for the ultra-trace

analysis of pesticide residues in real samples. In fact, preliminary results indicated that the SPE step alone cannot result in suitable preconcentration factors. Overall, SPE coupled with DLLME appears to be an excellent alternative extraction method for the determination of pesticides in complex food matrices, in that it is a sensitive, low cost, effective, and eco-friendly analytical method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.02.090>.

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