Microchemical Journal 98 (2011) 56–61

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Development of a headspace solid-phase microextraction/gas chromatography–mass spectrometry method for determination of organophosphorus pesticide residues in cow milk

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ARTICLE INFO

Article history:
Received 28 October 2010
Accepted 6 November 2010
Available online 24 November 2010

Keywords:
Milk
Organophosphorus pesticides
HS-SPME–GC/MS
Factorial design

ABSTRACT

In the past few years, organophosphorus compounds become one of the most widely used classes of pesticides due to their acute toxicity against a wide variety of pests. In this work, a method based on solid-phase microextraction in mode headspace (HS-SPME) coupled to gas chromatography–mass spectrometry (GC–MS) was developed and optimized through multivariate factorial design to determine residues of organophosphorus pesticides in cow's milk. Different parameters of the method were evaluated, such as fiber type, temperature, extraction and desorption times, sample volume, effect of salt addition and stirring velocities. The evaluated pesticides were dichlorvos, sulfotep, demeton-S, dimpylate, disulfoton, parathion, methyl parathion, fenitrothion, chlorpyrifos and ethion. The best results were obtained using polydimethylsiloxane/divinylbenzene fiber and headspace mode at 90 °C for 45 min, along with stirring at 600 rpm and desorption for 5 min at 250 °C. Under the optimized conditions, the proposed methodology was able to determine all of the pesticides with variation coefficients between 6.1% and 29.5%. Detection and quantification limits ranged from 2.16 to 10.85 μg L^{-1} and from 6.5 to 32.9 μg L^{-1}, respectively. To evaluate residues of these pesticides in milk, cows were exposed to the pesticides of interest and milk was collected after 24 h. The developed method was able to detect trace amounts of these pesticides in the collected milk samples.

1. Introduction

In the past few years, organophosphorus compounds have become one of the most widely used classes of pesticides in the world [1,2]. This class acts directly on the central nervous system, inhibiting the expression of the acetylcholinesterase enzyme [3], and possesses acute toxicity against a wide variety of insects and arthropods. They also present a relatively low persistence in the environment, which represents great progress in comparison to the organochlorine pesticides, in spite of organophosphorus pesticides being much more poisonous. Some compounds have also been observed to accumulate in adipose tissue, but they decompose within a few days or weeks [4].

Among foods, milk [5] stands out as participant at the top of the trophic food chain and as biomarker of environmental pollution. Milk is a complex biological matrix due to its high water, fat and protein content [6,7]. These characteristics usually demand special procedures for sample preparation to achieve an excellent analytical determination [8–10].

Therefore, it is necessary to develop new analytical methods that allow manipulation of complex matrices in a simple and efficient way. Liquid-phase microextraction (LPME) and solid-phase microextraction (SPME) are examples of promising new analytical techniques [11–15]. SPME [16] has proven to be a powerful and useful technique to meet these needs, and it has emerged as a versatile alternative method of analyte extraction and preconcentration, which requires little or no organic solvents—thus does not generate poisonous residues—is easily automated, and can also improve the limit of detection. SPME encompasses sampling, extraction, preconcentration and introduction of the sample into the analytical system in a single uninterrupted process, thus avoiding contamination of the matrix.

SPME is a miniaturized technique, in which the extraction and concentration processes of the analytes are carried out in dimensions that are different from solid phase extraction (SPE). In the first stage of SPME, a fiber of fused silica covered with a film of selective polymeric

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0026-265X
doi:10.1016/j.microc.2010.11.002

liquid, a solid phase, or both, is put in contact with the sample, which then results in partitioning or adsorption of the analyte between the matrix and the stationary phase. Soon afterwards, the fiber is transferred to an analytical instrument where the analytes are desorbed, separated and quantified [17–19].

SPME extraction can be performed either in the headspace (HS) or through direct insertion of fiber into the sample. In the first case, the sample is heated, the analytes transported to the vapor phase and then sorbed on the fiber, which is exposed directly to the vapor phase and thus does not stay in contact with the matrix [20–23].

Therefore, HS-SPME is an attractive alternative for the extraction of organophosphorus compounds in complex matrices, such as milk, as it is simple, fast and possesses low manipulation of the sample and high sensitivity [24]. However, there are several parameters that should be optimized to obtain a greater efficiency in the extraction of organophosphorus compounds, including fiber type, the time and temperature of extraction, sample volume added to the extraction flask and time and temperature of desorption, among others [25,26].

Furthermore, gas chromatography coupled to mass spectrometry (GC/MS) is a powerful tool to separate, identify and quantify volatile organic compounds in the most types of complex matrices.

The purpose of this study was to develop a method, based on HS-SPME–GC–MS, to identify and determine residues of organophosphorus pesticides in unprocessed cow milk, due to its importance as an indicator of environmental contamination.

2. Experimental

2.1. Reagents and solutions

Standard solutions of 1000 μg mL^{-1} for demeton-S, sulfotep, dimpylate, disulfoton, methyl parathion, parathion, dichlorvos, fenitrothion, chlorpyrifos and ethion were acquired from Absolute Standard, Inc (Hamden, USA). The solvents n-hexane and ethanol were of HPLC grade and were purchased from J.T. Baker (USA). Analytical grade reagents and deionized water were employed in all experiments.

A working solution of 100 μg mL^{-1} of the various organophosphorus pesticides was prepared by dilution of 1.0 mL of concentrated standard solution in a 1:1 n-hexane:ethanol solution. The final volume was adjusted to the mark in a 10.0 mL volumetric flask. Other organophosphorus solutions were prepared by further dilution in a similar way. All organophosphorus solutions were protected from light and stored at 5 °C before use.

2.2. Apparatus

The analyses were performed using a GC–MS system (Shimadzu GC-2010/QP-2010 high performance quadrupole, Kyoto, Japan) equipped with a split/splitless injector in the splitless mode and at 250 °C during the chromatographic run. Pesticides were separated in a capillary column (HP-5 MS 5%-phenyl-methylpolysiloxane; 30 m × 0.25 mm ID × 1.00 μm, Agilent, Palo Alto, USA) using helium (99.99%) as carrier gas at a 1.19 mL min^{-1} flow rate. The oven temperature was varied as follows: 60 °C (2 min), then warmed to 160 °C at 15 °C min^{-1} (hold 6 min), then warmed to 280 °C at 20 °C min^{-1} and held at 280 °C for 10 min.

The mass detector conditions were: transfer line temperature of 270 °C, ion source temperature of 260 °C and ionization mode with electron impact at 70 eV. The analysis was done in the SIM (selected ion monitoring) mode.

The HS-SPME procedures were carried out with a manual holder (Supelco, Bellefonte, USA) using polydimethylsiloxane (PDMS, 100 μm), polydimethylsiloxane divinylbenzene (PDMS-DVB, 65 μm) and polyacrylate (PA, 85 μm) fibers (Supelco, Bellefonte, USA) previously conditioned following manufacturer recommendations.

2.3. Milk samples

To develop the method, pasteurized milk (type C) was acquired in a local market and stored in the freezer (−18 °C) until analysis. To apply the method, unprocessed milk samples were collected at the Experimental Station of Aramari, located in the city of Aramari (12° 05′ S; 38° 30′ E; 200 m height) and whose cows had not been exposed to organophosphorus pesticides over the past three years.

Eight cows were then sprayed with organophosphorus-based pesticides for ticks (*Boophilus microplus*) and horn fly (*Haematobia irritans*) with four different products that are largely used in the region while two other cows were left as controls. The commercial products evaluated were *Ectofós* (chlorpyrifos and cipermetrin), *Colosso* (chlorpyrifos and dichlorvos), *Ciperthion* (ethion and cipermetrin) and *Carbeson* (dichlorvos and chlorfenvinphos).

During the experiment, a 50 mL aliquot of milk was collected daily in the morning, for seven consecutive days, at the time of milking, and immediately placed in the freezer. The frozen milk samples were transported to the laboratory for further analysis.

2.4. HS-SPME procedure

An aliquot of defrosted milk (12 mL), spiked with a known amount of organophosphorus standard (0.50 mg L^{-1}) was put in a sealed 20 mL glass vial. After waiting a period of 30 min to attain gas–liquid equilibrium, the vial septum was bored and PDMS-DVB fiber was exposed to the headspace. The extraction was performed by placing the vial into an aluminum heating block (4 cm in height by 14 cm in diameter) on a temperature-controlled heating plate (90 °C) with 600 rpm agitation for 45 min. Following extraction and pre-concentration, the fiber was then inserted directly into the GC injector for desorption at 250 ° C over 5 min.

2.5. HS-SPME multivariate optimization

The efficiency of the HS-SPME can be related to the following parameters: fiber material, temperature, extraction time interval, sample amount, desorption time and headspace equilibrium time. In this way, optimization of the microextraction conditions was a multiparameter evaluation task that could be overcome by multivariate techniques [27,28].

To identify relevant parameters that contributed to the sensitivity of the proposed HS-SPME method, a factorial screening design was carried out. Factorial design is a valuable tool for simultaneous investigation of the effect of several variables over the evaluated response (*e.g.*, analytical sensitivity) requiring a reduced number of experiments. In the initial step, a screening of 2^{7-3} fractional factorial design using a linear multivariate regression model [29,30] was developed. The variables evaluated by the screening experimental design were extraction temperature, extraction time, headspace

Table 1
Experimental values of variables evaluated by the 2^{7-3} factionary factorial design.

Variable	Coded level	
	−1	1
Extraction temperature, °C	50.0	90.0
Extraction time, min	30.0	60.0
Equilibrium time, min	10.0	30.0
Desorption time, min	1.0	5.0
Sample volume, mL	6.0	12.0
NaCl, mg	0.0	5.0
Stirring speed, rpm	300.0	600.0

Table 2
Selected ions and time windows for GC–MS analysis of the selected pesticides.

Window	Acquisition time, min	Organophosphorus compound	Most abundant ion	Reference ions
1	3:51–15:50	Dichlorvos	220	109 and 185
		Sulfotep	322	202 and 266
2	15:51–18:20	Demeton-S	88	170 and 171
		Dimpylate	304	137 and 179
		Disulfoton	274	88 and 89
		Methyl Parathion	263	109 and 125
3	18:21–19:30	Fenitrothion	277	260 and 278
		Chlorpyrifos	314	197 and 199
		Parathion	291	109 and 292
4	19:31–30:00	Ethion	384	153 and 231

equilibrium time, desorption time, sample volume, effect of salt addition and stirring speed. The values investigated in these experiments and their coded levels are listed in Table 1. The response evaluated during all experiments was the total sum of organophosphorus peak areas, obtained by GC–MS for the evaluated organophosphorus pesticides.

Once statistically significant variables were obtained, the Response Surface Methodology associated with the factorial design was employed to highlight the optimized conditions. The statistical experimental designs and optimization calculations were performed using the Statistica 7.0 (Statsoft, USA) software package.

3. Results and discussion

3.1. Chromatographic analysis

Peak retention times and resolutions were optimized in the full scan mode using a 1.00 μg mL^{-1} standard solution and varying the oven temperature and carrier gas flow rate. In these evaluations, the characteristic ions were chosen for quantification of each pesticide.

Matrix components could provide variation in the detector response to pesticides. Thus, the matrix effect was evaluated by comparing the detector responses of pesticide standards prepared in 1:1 n-hexane:ethanol with those of standards prepared in pasteurized milk. When standards were prepared by spiking blank pasteurized milk samples with known amounts of pesticides, higher peak areas were obtained for each concentration. Consequently, the quantification of pesticide residues was carried out using matrix-matched standards. The CG–MS based on the SIM mode was developed using the most intense ion and two other auxiliary ions for each pesticide, as described in Table 2. Fig. 1 shows a typical chromatogram obtained for organophosphorus pesticides in a spiked milk sample using GC/MS in SCAN mode.

3.2. Fiber selection

The logarithm water–octanol partition coefficient (log K_{ow}) of the pesticides is related to their affinities with the hydrophilic and lipophilic layers. For the compounds studied, the log K_{ow} varied between 1.32 (demeton-S) and 5.07 (ethion) with the majority of compounds with values greater than 3.0 (Table 3), showing that the ten compounds studied had considerable variation in polarity. For more efficient extraction of these pesticides from the milk matrix, it was thus necessary to evaluate fibers with different polarities.

Three fiber phases were tested, a polar phase (polyacrylate—PA), a non-polar phase (polydimethylsiloxane—PDMS) and a bipolar phase (polydimethylsiloxane/divinylbenzene). The evaluation of the efficiency of each phase was performed by comparing the total average signal (n = 5) obtained for the peak areas detected for each pesticide. The results obtained are illustrated in Fig. 2.

Except for disulfoton and ethion, which showed better results with the nonpolar phase (PDMS), the bipolar phase (PDMS-DVB) was the most efficient in the extraction of most compounds. Among the ten compounds studied, ethion (log K_{ow} = 5.07) was the least polar, giving the best extraction in the nonpolar phase. Better results were obtained using the PDMS-DVB fiber, results that agreed with those available in the literature [32].

3.3. Optimization of HS-SPME conditions

The results obtained from the evaluation of the significant parameters by factorial design are summarized in the Pareto chart of effects, depicted in Fig. 3. As can be seen, the extraction temperature, NaCl mass and milk volume were significant at a 95% confidence level. The results showed that increasing the extraction temperature and sample volume or reducing the mass of added NaCl would result in greater peak areas. For further experiments NaCl was suppressed. Therefore, only extraction temperature and time were evaluated for optimization purposes.

The response surface graph (Fig. 4) obtained from the results of the fractional factorial design also exhibited the relationship between the

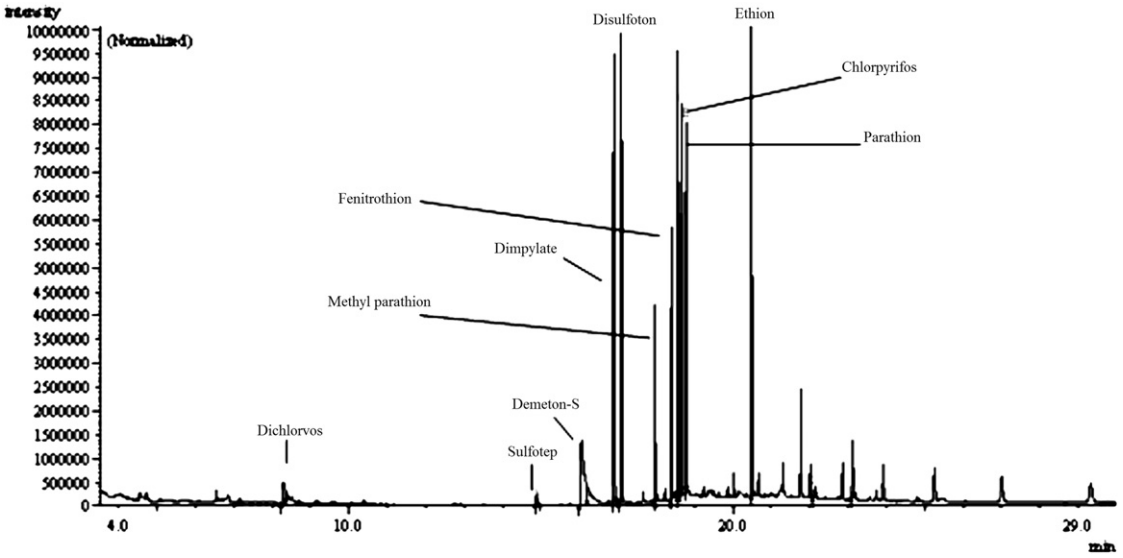

Fig. 1. Chromatogram obtained by HS-SPME/GC–MS analysis of an organophosphorus-spiked milk sample in SCAN mode. The experimental conditions are presented in Section 2.

Table 3
Molecular weight, log K_{ow} and vapor pressure of the studied pesticides.
Source: IUPAC [31].

Pesticides	MW	Log K_{ow}	VP/mPa (20 °C)
Dichlorvos	221.0	1.90	2100
Sulfotep	322.3	3.99	14
Demeton-S	230.3	1.32	40
Dimpylate	194.2	3.81	1300
Disulfoton	274.4	3.95	13
Methyl parathion	263.2	3.00	0.2
Fenitrothion	277.2	3.43	18
Chlorpyrifos	350.6	4.69	2.7
Parathion	291.3	3.83	0.89
Ethion	384.5	5.07	0.2

increase of milk sample volume and extraction temperature with higher responses (*e.g.*, analytical sensitivity).

In spite of these findings, considering that extraction temperatures higher that 90 °C would lead to deleterious effects on the milk matrix, and sample volumes higher than 12 mL would increase the risk of direct contact of the milk sample with the SPME fiber, it was decided to maintain the temperature and volume of milk at the highest studied levels and suppress the use of NaCl. Thus, according to the results obtained in the multivariate optimization and considering the peculiarities of the milk matrix, the best conditions for extraction of organophosphorus compounds from milk by HS-SPME are listed in Table 4.

3.4. Figures of merit

The repeatability of the proposed method was evaluated by carrying out seven replicates of the HS-SPME/GC–MS procedure using a sample of pasteurized milk previously spiked with 0.500 mg L^{-1} of organophosphorus pesticides. The coefficients of variation obtained ranged from 6.15% (sulfotep) to 29.25% (demeton-S), which are acceptable values, considering the complexity of the sample matrix, while linear correlation coefficients of analytical curves ranged from 0.9253 (fenitrotion) to 0.9987 (dimpylate). The limits of detection and limits of quantification were calculated as follows [33,34]:

$$\mathrm{LOD} = 3 \times \frac{s}{a} \quad (1)$$

$$\mathrm{LOQ} = 10 \times \frac{s}{a} \quad (2)$$

where s is the standard deviation of the linear coefficient from the analytical curve and a is the angular coefficient from the analytical curve. The limits of detection, quantification and the absolute recoveries obtained by HS-SPME in pasteurized milk are presented in Table 5.

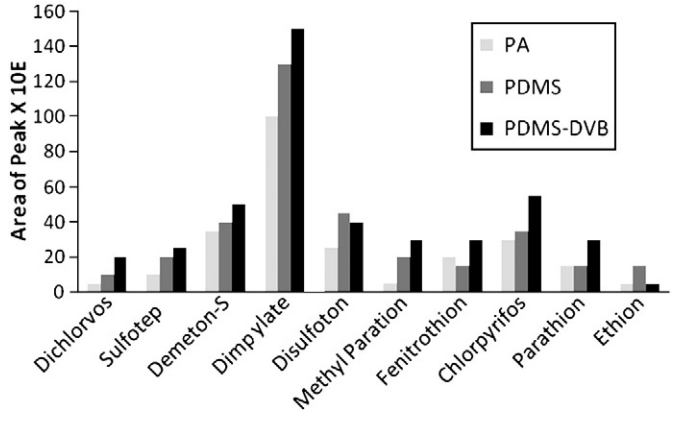

Fig. 2. Evaluation of the efficiency of different types of fiber used for microextraction of organophosphorus pesticides.

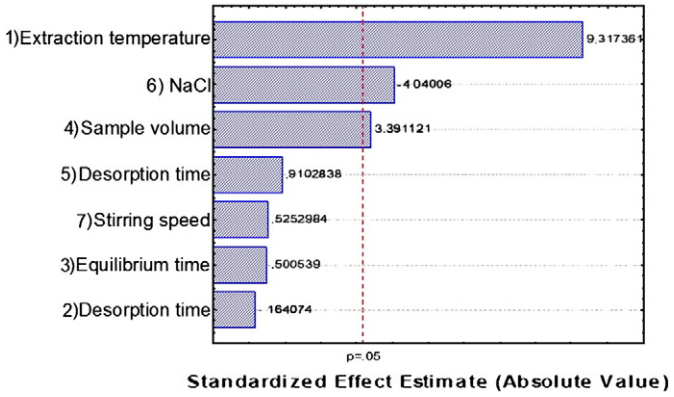

Fig. 3. Pareto chart of effects for 2^{7-3} fractional factorial design.

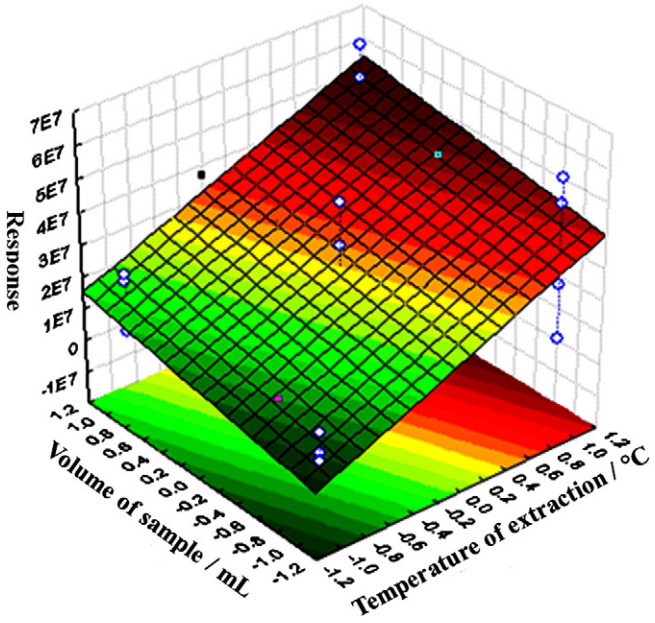

Fig. 4. Response surface obtained for the factorial experimental design.

Because SPME is a non-exhaustive extraction technique, recoveries are usually low. In this work, the recoveries found were comparable to, or even higher than, those reported in the literature, where available, except for ethion [36,37].

Based on the values of the octanol–water partition coefficients ($\log K_{ow}$) which measure the hydrophilicity or lipophilicity properties of each pesticide, the low recoveries of ethion ($\log K_{ow} = 5.07$) are explained by its higher affinity for the sample matrix, rich in lipids, than for the fiber [38].

Table 4
Optimized values for the variables, obtained from the response surface method.

Optimized conditions	
Extraction temperature (°C)	90
Extraction time (min)	45
Equilibrium time (min)	30
Desorption time (min)	5
Milk volume (mL)	12
NaCl mass (mg)	0
Stirring speed (rpm)	600

Table 5
Limits of detection, quantification and absolute recoveries obtained by HS-SPME/GC–MS.

Organophosphorus compound	Limit of detection ($\mu g\ L^{-1}$)	Limit of quantification ($\mu g\ L^{-1}$)	Average absolute recovery (n = 3) (%)	Absolute recovery from the literature (%) [35]
Dichlorvos	3.8	11.4	0.05 ± 0.01	
Sulfotep	3.6	10.9	0.70 ± 0.30	
Demeton-S	2.9	8.9	0.24 ± 0.01	
Dimpylate	2.8	8.5	0.18 ± 0.07	0.31
Disulfoton	4.1	12.4	0.09 ± 0.01	
M Parathion	10.9	32.9	0.20 ± 0.10	0.16
Fenitrothion	4.8	14.6	0.15 ± 0.05	
Chlorpyrifos	4.5	13.7	0.30 ± 0.20	0.11
Parathion	4.7	14.1	0.18 ± 0.07	
Ethion	2.2	6.5	0.05 ± 0.02	0.32

Table 6
Linear range and R^2 obtained for the proposed method.

Organophosphorus compound	Linearity ($\mu g\ L^{-1}$)	R^2
Dichlorvos	11.4–32.0	0.9902
Sulfotep	10.9–56.0	0.9874
Demeton-S	8.9–32.0	0.9862
Dimpylate	8.5–56.0	0.9987
Disulfoton	12.4–32.0	0.9900
Methyl Parathion	32.9–56.0	0.9787
Fenitrothion	14.6–32.0	0.9523
Chlorpyrifos	13.7–48.0	0.9944
Parathion	14.1–28.0	0.9720
Ethion	6.5–32.0	0.9875

The linear range of the proposed method was evaluated using a standard solution prepared in pasteurized milk free of organophosphorus pesticides. The results are listed in Table 6.

3.5. Application of the developed method

The determination of pesticide residues in milk, from cows exposed to organophosphorus-based pesticides, using as blank the milk of control cows, was carried out using the proposed method. Chlorpyrifos residues were found in the milk of four cows sprayed with formulations containing this compound, while ethion residues were also detected in the milk of two cows sprayed with this pesticide. The results obtained have shown that, within a period of 24 h, it was possible to detect residues of chlorpyrifos at concentrations below its limit of quantification. In regards to ethion, this pesticide could be detected at concentrations below its limit of quantification up to 72 h after the spraying of cows.

4. Conclusions

A method based on HS-SPME, presented here for the analysis of residues of organophosphorus pesticides in cow's milk, was successfully optimized. At optimized conditions the limits of detection and quantification ranged from 2.16 $\mu g\ L^{-1}$ and 6.55 $\mu g\ L^{-1}$ for ethion to 10.9 $\mu g\ L^{-1}$ and 32.9 $\mu g\ L^{-1}$ for Methyl Parathion. When applied, the proposed method was able to detect chlorpyrifos and ethion residues in samples of unprocessed milk. Furthermore, the method allowed the detection of chlorpyrifos residues even 24 h after spraying the cows, while ethion residues were detected up to 72 h after spraying. Finally, the feasibility of the proposed method was demonstrated by its low cost and its no need for solvent use in the extraction step, in agreement with the principles of the Green Chemistry.

It should be emphasized that this method could also be optimized for the analysis of pesticide residues in many other biological and environmental matrices as well.

Acknowledgements

The present work was supported by the Brazilian National Research Council (CNPq), the State of Bahia Foundation for Support to Research (FAPESB), PRONEX, FINEP and CAPES. The authors also thank the Company for the Agricultural Development of Bahia (EBDA).

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