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Short communication

Determination of quinalphos in blood and urine by direct solid-phase microextraction combined with gas chromatography–mass spectrometry

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Abstract

A new method based on direct solid-phase microextraction (DI-SPME) followed by gas chromatography–mass spectrometry was developed for the purpose of determining quinalphos in blood and urine. Two types of coated fibre have been assayed and compared: carbowax™/divinylbenzene (CW/DVB 65 μm) and polydimethylsiloxane (PDMS 100 μm). The main parameters affecting the SPME process such as temperature, salt addition, pH, stirring and adsorption/desorption time profiles were optimized to enhance the sensitivity of the procedure. The method was developed using only 100 μL of blood and urine. Limits of detection of the method for blood and urine matrices were, respectively, 10 and 2 ng/mL. Linearity was established over concentration ranges from 0.05 to 50 μg/mL for blood, and 0.01 to 50 μg/mL for urine, with regression coefficients ranging between 0.9991 and 0.9999. Intra- and interday precision values were less than 13%, and accuracy was within ±15% of the nominal concentration for all studied levels in both matrices. Absolute recoveries were 14 and 26% for blood and urine, respectively.

Keywords: Solid-phase microextraction; Quinalphos; Gas chromatograpy

1. Introduction

Quinalphos (*O*,*O*-diethyl *O*-quinoxalin-2-yl phosphorothioate) is an ester of phosphorothioic acid [1]. It was first introduced in Portugal in 1986 (Ekalux®), and nowadays is the primary agent responsible for intoxications with organophosphorous insecticides in this country.

It is effective against both biting and sucking pests on vegetables, especially against the diamond-back moth, and also in the control of mosquitoes and mites [2,3].

Organophosphorous compounds are by far the most important class of pesticides, both in terms of worldwide usage and their toxicity to humans [4].

These compounds are usually well absorbed by the gastrointestinal tract, but also by the skin and airways [5,6]. Like other organophosphorous insecticides, quinalphos acts by inhibiting acetylcholinesterase (AChE) in the nervous tissue. This enzyme is responsible for the destruction and termination of the biological activity of the neurotransmitter acetylcholine (ACh) [1,6,7]. Build-up of ACh at the neural junction leaves the muscles, glands and nerves in a constant state of stimulation, which produces a wide range of acute symptoms, such as dizziness, confusion and blurred vision, etc. Severe poisoning leads to coma, breathing difficulties, cyanosis and cardiac arrhythmias [4–7].

Solid-phase microextraction (SPME) was developed by Arthur and Pawliszyn in the early 1990 at the University of Waterloo (Ontario, Canada) [8], and has a wide range of applications, namely in the determination of drugs of abuse [9–12], medical substances [13,14], pesticides [15–19] and more recently strychnine [20].

The determination of quinalphos can be accomplished by means of liquid–liquid extraction [21–23], matrix solid-phase dispersion [24,25], solid-phase extraction [26] or SPME. This latter technique has been applied to the determination of quinalphos mainly in agricultural samples [27,28], using the

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direct immersion (DI) approach. However, no data is yet available on the application of DI-SPME to determine quinalphos in blood and urine specimens. In fact, only one paper describes the determination of this pesticide in blood, but using the headspace sampling technique [29].

This paper describes a new and sensitive method based on DI-SPME coupled to gas chromatography/electron impact ionisation–mass spectrometry (GC/EI–MS) to determine quinalphos in whole blood and urine utilizing only 100 μL of sample.

2. Experimental

2.1. Reagents and equipment

Analytical standards, quinalphos and ethion (Fig. 1), were purchased from Riedel-de Haën (Seelze, Germany) and Polyscience Corp. (Niles, IL, USA), respectively. Ethion, an organophosphorous insecticide not commercially available in Portugal, was used as internal standard (IS). Both pesticide standards were of 98–99% purity.

Methanol (HPLC grade), phosphoric acid, sodium hydrogenphosphate and sodium chloride (analytical grade) were obtained from Merck Co (Darmstadt, Germany).

Methanolic standard stock solutions at 1000 μg/mL were prepared and stored at −20 °C. Subsequently working solutions at 100, 10 and 1 μg/mL for quinalphos and at 100 μg/mL for ethion were prepared in methanol, and stored protected from light at 4 °C.

Phosphate buffer solutions (0.07 M) were prepared by mixing solutions of phosphoric acid and sodium hydrogenphosphate, in variable proportions, according to the desired pH [30].

Fresh human blood was obtained from the excess supplies of the Portuguese Institute of Blood (outdated transfusions), preserved with citrate phosphate dextrose (1:7), and urine samples were obtained from healthy laboratory staff.

The SPME fibre holder for manual use and the coated fibres (100 μm Polydimethylsiloxane (PDMS) and 65 μm Carbowax^{TM}/Divinylbenzene (CW/DVB)) were obtained from Supelco (Bellefonte, PA, USA).

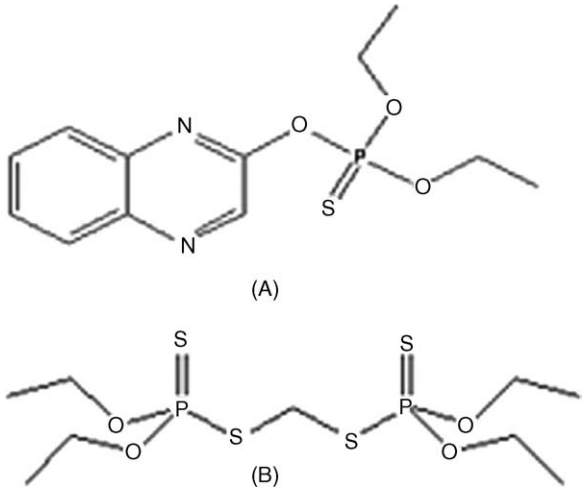

Fig. 1. Molecular structures of quinalphos (A) and ethion (B).

Chromatographic analysis was performed using a 6890 Series gas chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a model 5973 mass selective detector (Hewlett-Packard, Waldbronn, Germany). A capillary column (12 m × 0.25 mm I.D., 0.25 μm film thickness) packed with 5% phenylmethylsiloxane (Ultra 2), supplied by J & W Scientific (Folsom, CA, USA), was used.

The GC oven was kept at 100 °C for 2 min after which the temperature was raised by 10 °C min^{-1} to 200 °C, and finally by 24 °C min^{-1} to 270 °C, where it was kept constant for 2 min. The injector port and the detector temperatures were set at 240 and 280 °C, respectively. Splitless injection mode was adopted, and helium, with a constant flow rate of 1 mL min^{-1}, was used as the carrier gas.

The mass spectrometer was operated with a filament current of 300 μA and an electron energy of 70 eV in the electron impact (EI) mode. Quantitation was done in the selected ion monitoring (SIM) mode, and the ions were monitored at *m/z 146*, 157 and 118 for quinalphos, and at *m/z 231*, 153 and 125 for ethion (quantitation ions are italicized).

The retention times were 12.84 and 14.56 min for quinalphos and ethion, respectively, obtaining a good separation of both compounds.

2.2. Fibre conditioning

New fibres were conditioned in the injector of the GC system as follows: PDMS fibres were heated at 250 °C for 30 min and CW/DVB at 220 °C for 30 min, according to the suppliers' specifications. The fibres were also cleaned every day prior to the first extraction by desorption in the injection port for 2 min.

2.3. Extraction procedure

All the extractions were performed in a 1.5 mL sample vial (40 mm × 7 mm), in order to keep the headspace volume to a minimum. The fibre was directly immersed in the sample with a depth of 1 cm.

The parameters that could affect the SPME process were optimized in a preliminary study (Section 3.1), and the final conditions were as follows.

2.3.1. SPME procedure for blood

For sample preparation was added 1 μg of IS to 100 μL of blood, and a final volume of 1 mL was obtained with water. The sample was then agitated for 30 s, and a CW/DVB coated fibre was directly immersed in it for 60 min at 60 °C. After extraction, the fibre was washed by immersion in deionised water for 5 s, and thermally desorbed in the injection port of the GC system for 1 min, after which the chromatographic run was started.

2.3.2. SPME procedure for urine

The SPME procedure for urine samples was similar to that of blood, except that the extraction was performed at 90 °C instead of 60 °C, and the washing step was not necessary.

The washing step prior to desorption had an important role in preventing protein burden in the fibre coating when extracting blood samples. This way, the coated fibres lasted for more than 100 reproducible extractions, both in urine and blood samples.

3. Results and discussion

3.1. Optimization of SPME

The different parameters that can affect the SPME process, such as type of coated fibre, adsorption and desorption time profiles, temperature, agitation, salt addition and pH were optimized for each particular sample (blood and urine), analyzing samples spiked with 1 μg/mL of quinalphos and ethion ($n = 3$).

3.1.1. Selection of fibre coating

The choice of an appropriate coating is essential for the SPME method because the extraction performance of each fibre is different depending on the molecular mass and the polarity of the analytes to be extracted. Two types of fibre coating were tested: the 100 μm PDMS and the 65 μm CW/DVB. To select the best coated fibre, samples of blood and urine were spiked with quinalphos at 1 μg/mL, and were extracted using both fibres. After 40 min of extraction, the fibres were retracted and subjected to desorption in the injector port of the GC for 3 min, and the obtained chromatograms were compared (in terms of peak area of quinalphos). For each fibre, the measured peak area in the chromatogram was compared with that obtained by direct injection of 1 μL of a methanolic solution with the same concentration. CW/DVB fibre extracted about three times more analyte than PDMS in both matrices, and therefore the latter was excluded from the study.

3.1.2. Adsorption and desorption times

Solid-phase microextraction is an equilibrium process that involves partitioning of analytes between two phases: an aqueous or gaseous phase (the sample) and a solid-phase (the fibre coating). Therefore, the optimization of the contact time between these two phases is crucial. To find the best adsorption time, the fibre was directly immersed in the samples (blood and urine spiked with 1 μg/mL of quinalphos) for 1, 5, 10, 15, 25, 35, 40, 60, 90 and 180 min, followed by a desorption of 3 min at 240 °C in the injector port of the CG. The extraction time profile was established by plotting the area counts against the extraction time. As can be seen in Fig. 2, equilibrium was not reached even after 180 min of contact, and therefore it was necessary to establish a good compromise between extraction yield and extraction time. This way, an extraction time of 60 min was chosen for each matrix. This was possible because SPME is a quantitative method at every time of the extraction time profile [31], and sensitivity was sufficient prior to equilibrium. Longer adsorption times were not chosen because the advantages of the technique would be wasted (one advantage of SPME lays in its speed).

The optimization of desorption time aims at achieving the complete desorption of the adsorbed analyte, to improve sensitivity, and simultaneously avoid carry-over effects.

After extraction of spiked blood and urine samples for 60 min, the fibre was subjected to desorption at 240 °C in the injection port of the GC for 1, 2, 3, 4 and 6 min. Peak areas obtained for each desorption time were compared. A 1 min desorption yielded the highest peak areas, and therefore this was the selected time.

To test for carry-over effects, samples containing high amounts of quinalphos (100 μg/mL) were extracted for 60 min, and the fibre was subjected to the desorption process in the hot injector of the GC for 1 min. After the chromatographic run, a second desorption of the fibre was performed at the same temperature, to test for remains of the pesticide that would contaminate further analyses. No peaks were observed after the second desorption, and therefore the selected time for desorption was 1 min.

3.1.3. Temperature effect

With the purpose of enhancing the extraction yield, it is common to heat the samples when working with SPME, mainly in the headspace sampling technique. To evaluate the temperature effect on the extraction yield, spiked samples of blood and urine were extracted at different temperatures (room temperature, 40, 50, 60, 70, 80 and 90 °C for urine samples; and room temperature, 40, 50, 60 and 70 °C for blood samples) for 60 min.

We observed that the extraction yield increased with the temperature increase (Fig. 3), and the chosen temperatures were 90 °C for urine and 60 °C for blood. A 60 min extraction of

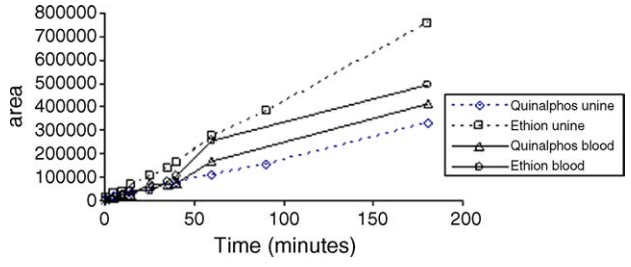

Fig. 2. Plot of peak areas of the analytes vs. the extraction time in blood and urine.

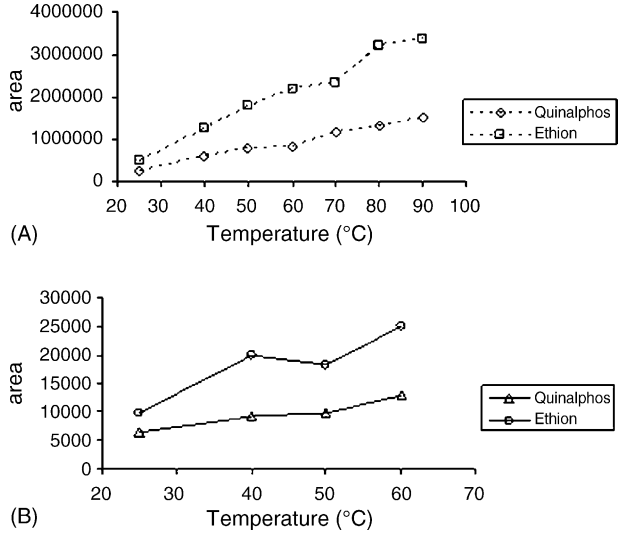

Fig. 3. Plot of peak area of the analytes vs. the extraction temperature in urine (A) and in blood (B).

urine samples at 90 °C showed even better results than 180 min at room temperature. Furthermore, at 90 °C, the extraction yield of quinalphos from urine was increased by a factor of 7, when compared to room temperature extraction yield. Blood samples could not be heated above 60 °C because of coagulation (we experienced coagulation of the samples at 70 °C), and a 60 min extraction at this temperature yielded similar amounts of the pesticide when compared to a 180 min extraction at room temperature.

In equilibrium, the temperature increase during extraction affects negatively the extraction recovery because de distribution constant between the sample and the fibre coating decreases. However, in non-equilibrium conditions, it is possible to improve sensitivity with increasing the temperature at which the extraction is performed [31]. This fact may explain our results, since the time selected for extraction was shorter than the equilibrium time for quinalphos (as above mentioned, equilibrium was not reached even after 180 min).

3.1.4. Effect of agitation

To evaluate the influence of agitation on the extracted amount of quinalphos, spiked blood and urine samples at 1 μg/mL were analyzed with, and without agitation during the adsorption process. Agitation was performed by means of a magnetic stirrer at 1250 rpm. Peak areas obtained with, and without stirring were compared. Sample agitation during adsorption did not improve significantly the extraction yield in both matrices, and therefore it was decided not to agitate the samples, as it would make the process more complicated.

3.1.5. Effect of salt addition and pH modification

To enhance the extraction of organic analytes from aqueous matrices, it is common to use pH adjustment and salting. The pH of the sample has an important role in SPME, as it affects acid–base equilibrium between the ionized species of the analyte, and hence the extraction yield. The effect of pH on the extraction yield was evaluated by diluting spiked blood and urine samples at 1 μg/mL in phosphate buffer 0.07 M (pH 5–8) instead of water. The peak areas obtained for each of the pH values were compared. The pH of the sample did not affect significantly the extraction of quinalphos from a blood sample, and the results were similar to those obtained by diluting the sample with water. On the other hand, a slight decrease in the extracted amount was observed with increasing pH in urine, and the best results were obtained after diluting the sample with water. This may have been due to a possible effect of an increase in salt concentration derived from the phosphate buffer. The effect of ionic strength on extraction efficiency was evaluated by analyzing the amount of quinalphos extracted in sample solutions containing 0, 0.375, 0.75, 1.5, 3, 5 and 10% of sodium chloride. The extracted amount decayed dramatically with the increase of salt concentration in the sample, which corroborates the results obtained in the pH experiments. Fig. 4 shows the influence of salt addition on the extraction yield for both matrices.

Therefore, this study was performed without salt addition or pH changes.

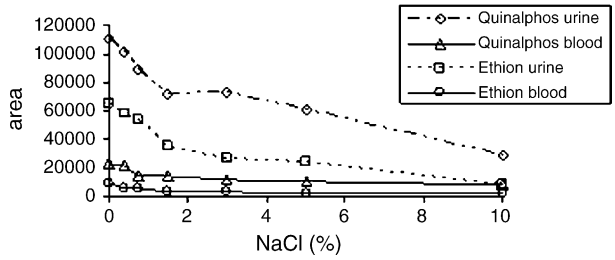

Fig. 4. Plot of peak areas of the analytes vs. salt added in the sample prior to SPME extraction.

3.2. Validation procedure

Method validation was performed in order to study selectivity, linearity, precision and accuracy (intra and interday), recovery, limits (of detection and quantitation) and stability, according to internationally accepted criteria [32,33].

Selectivity was examined by analyzing 10 blank blood and urine samples (of different origin), and comparison of the obtained chromatograms with those obtained spiking the samples with both compounds at low concentrations (0.01 μg/mL for urine, and 0.05 μg/mL for blood). The influence of other substances that might eventually be present (other organophosphorous compounds, pyrethroids, caffeine, etc), was also evaluated by spiking urine and blood samples with high concentrations of these compounds and low amounts of the analyte, and could be excluded due to different retention times and/or mass spectra. As can be seen in Fig. 5, no interferences were observed.

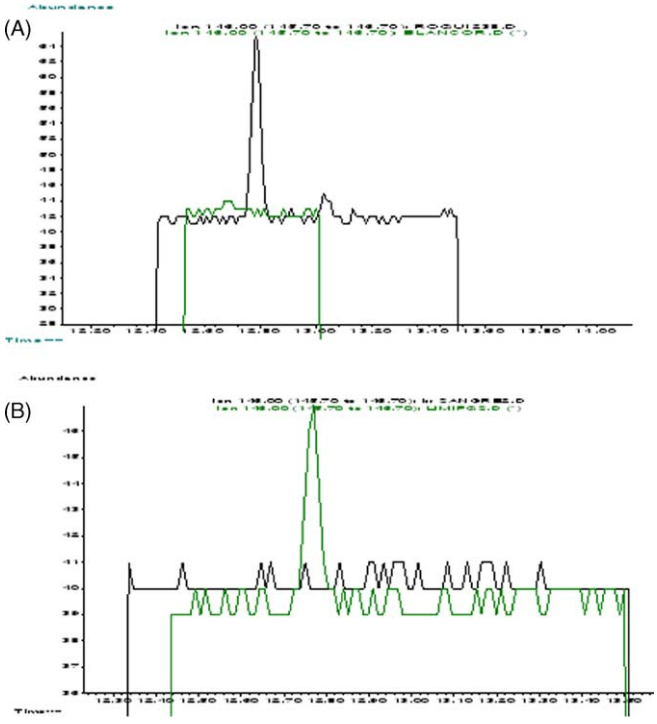

Fig. 5. Merged fragmentograms (ion 146) obtained from urine (A) and blood (B) samples spiked with a low concentration of quinalphos (10 and 50 ng/mL, respectively) and from blank samples.

Table 1
Analytical performance of both methods for the analysis of quinalphos in blood and urine

Sample	Calibration range (μg/mL)	Linearity ($n = 7$)		R^2 values	LOD (ng/mL)	LOQ (ng/mL)
		Slope	Intercept			
Blood	0.05–5.00	0.0912 ± 0.0016	0.0091 ± 0.0027	0.9993 ± 0.0006	10	50
	5.00–50.0	0.1225 ± 0.0076	0.1654 ± 0.0148	0.9999 ± 0.0030		
Urine	0.01–0.50	0.0236 ± 0.0008	0.0001 ± 6.7 E-05	0.9998 ± 0.0002	2	10
	0.50–50.0	0.0318 ± 0.0008	0.0057 ± 0.0002	0.9991 ± 0.0002		

In order to determine calibration curves, two linear ranges were established for each biological specimen: 0.05–5.0 μg/mL (7 calibrators) and 5.0–50.0 μg/mL (6 calibrators) for blood, and 0.01–0.5 μg/mL (6 calibrators) and 0.5–50.0 μg/mL (12 calibrators). The calibrators were evenly distributed over the respective linear ranges, and were prepared and analyzed using the above mentioned procedures. The calibration curves were obtained by plotting the peak-area ratio between analyte and IS against analyte concentration, obtaining coefficients of correlation ranging from 0.9991 to 0.9999 (Table 1). Calibrator's accuracy was within ±15% of the nominal concentration (±20% at LLOQ) for all concentration levels, and was considered acceptable according to the FDA's guidelines for bioanalytical method validation [32].

Limit of detection (LOD) was defined as the lowest amount of quinalphos in the sample which could be detected but not necessarily quantitated, and yielding a signal-to-noise ratio of at least 3.

Limit of quantitation (LOQ) was defined as the minimum concentration of quinalphos that could be measured reproducibly and accurately (CV ≤20% and bias ±20% of the nominal concentration). These limits are presented in Table 1.

It should be stated that LOD and LOQ in blood were obtained utilizing only 100 μL of sample, and were close to analytical limits for quinalphos determination in blood using SPME that have been published elsewhere [29]. However, these published results refer to a sample volume of 500 μL, and the sampling technique was headspace-SPME. We believe that the use of a quite high temperature while extracting the blood samples have contributed to the low limits obtained. Regarding the results in urine, it is not possible to compare the limits, since the determination of quinalphos in this matrix is not published yet.

Intra- and interday precision and accuracy were estimated at several concentrations evenly distributed over the calibration range (0.25, 2.50, 10.0, 25.0 and 50.0 μg/mL for blood; and 0.05, 0.50, 2.50, 10.0, 25.0 and 50.0 μg/mL for urine), with six replicates for each level. For intraday precision, sets of samples spiked at the chosen concentrations were analyzed in the same day, and the correspondent coefficients of variation (CV, %) were calculated. Interday precision was assessed in a similar way, analyzing spiked samples on different days over a period of 10 months. The calculated CVs were less than 15% for all concentration levels. Furthermore, accuracy (bias, %) of intra- and interday precision assays was within ±15% of the nominal concentration (Table 2), fulfilling internationally accepted criteria [32–34].

For the determination of absolute recovery, blood and urine samples spiked with quinalphos at three concentration levels (0.50, 5.0 and 25.0 μg/mL) were analyzed, and peak areas were compared with those obtained by a splitless injection of 1 μL of a methanolic solution containing the same amount of the analyte. As can be seen in Table 2, recovery was sample dependent, and the higher recoveries were obtained in urine. In fact, blood is a more complex matrix, with a high content of proteins and other bio-molecules, which may impair the mass transfer of analyte from the sample to the fibre coating.

The obtained recovery values were higher than those normally seen in SPME methods [20], namely in the determination

Table 2
Precision, accuracy and recovery

Sample	Concentration (μg/mL)	Recovery (%)	Concentration (μg/mL)	Intraday precision			Interday precision		
				Concentration mean (μg/mL)	CV (%)	Bias (%)	Concentration mean (μg/mL)	C.V. (%)	Bias (%)
Blood	0.50	14.1	0.25	0.25	9.13	2.01	0.27	9.67	10.52
	5.0	16.9	2.50	2.54	4.22	1.63	2.36	8.60	−5.53
			10.0	10.47	8.33	4.73	9.56	7.89	−4.41
	25.0	12.5	25.0	23.46	7.86	−6.17	27.10	2.28	8.39
			50.0	53.36	5.63	6.70	45.09	6.67	−9.82
Urine	0.50	27.7	0.05	0.04	13.09	−14.61	0.05	4.68	1.10
			0.50	0.54	3.06	8.61	0.53	8.69	6.67
	5.0	26.6	2.50	2.35	4.02	−6.01	2.33	5.48	−6.66
			10.0	9.51	2.92	−4.86	9.78	2.15	−2.21
	25.0	24.5	25.0	24.73	3.05	−1.09	24.41	5.77	−2.35
			50.0	51.43	1.55	2.85	50.08	3.54	0.17

$n = 6$; bias = [(measured concentration − nominal concentration)/nominal concentration] × 100.

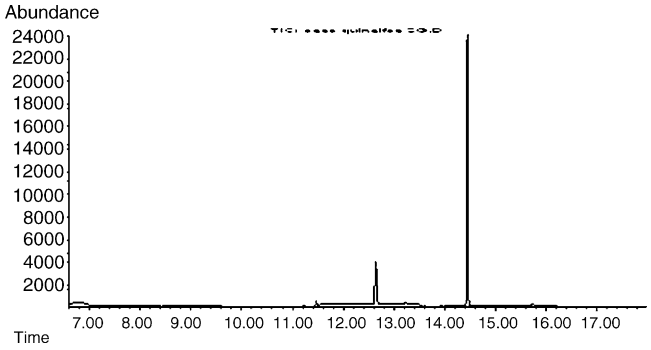

Fig. 6. Chromatogram corresponding to a real blood sample (case 21: 1.12 μg/mL).

of organophosphorous compounds in blood using the headspace sampling technique [29]. This may be explained by the effect of the temperature at which the extractions were performed. On the other hand, the results obtained for urine cannot be compared because no data is available on the determination of this pesticide in this specimen.

In order to study stability in processed samples at two concentration levels, blood and urine were spiked with 0.25 and 10 μg/mL of quinalphos, and extracted using the above mentioned procedures ($n = 3$). After extraction, the fibre was retracted, but desorption was not performed immediately; however, instead of that, the fibre was left standing for controlled time intervals (5, 10, 15, 20 and 30 min) before desorption. The measured concentrations for each tested time did not deviate more than 10% from the nominal concentration.

Short-term stability was evaluated at the same concentration levels ($n = 3$). Blood and urine were spiked with 0.25 and 10 μg/mL of quinalphos, and these samples were left at room temperature for 18 h. The measured concentrations did not deviate more than 10% from the nominal concentration.

Finally, the described methods were successfully applied to 36 authentic samples proceeding from medico-legal autopsies, as well as hospital cases, obtained from the Laboratory of Forensic Toxicology (Delegation of Coimbra, National Institute of Legal Medicine, Portugal). The results obtained by applying the method to these samples are shown in Table 3, and Figs. 6 and 7 show typical chromatograms of cases 21 and 36.

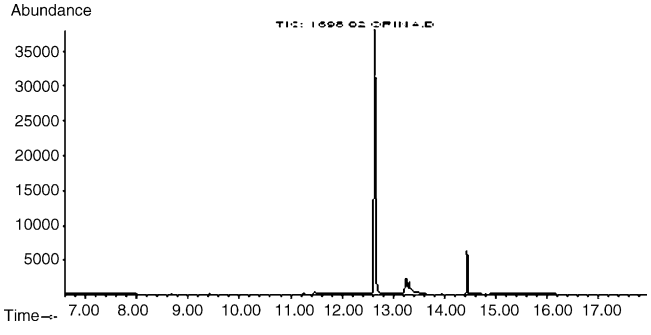

Fig. 7. Chromatogram corresponding to real urine sample (case 36: >50 μg/mL).

Table 3
Results obtained by applying the method to 36 real samples

Case	Concentration (μg/mL)	
	Blood	Urine
1	>50	–
2	0.37	–
3	0.34	0.28
4	0.12	–
5	0.14	–
6	31.65	0.35
7	34.23	–
8	3.95	–
9	0.48	–
10	>50	–
11	0.15	–
12	<LOQ	–
13	2.46	–
14	0.55	–
15	0.74	–
16	5.12	0.04
17	>50	–
18	0.68	0.12
19	<LOQ	–
20	<LOQ	0.03
21	1.12	–
22	0.52	–
23	0.93	–
24	0.55	–
25	0.20	–
26	0.13	–
27	0.99	0.29
28	0.41	–
29	0.82	–
30	0.43	–
31	0.20	1.29
32	0.08	–
33	0.09	–
34	<LOQ	–
35	1.96	0.42
36	0.78	>50

4. Conclusions

DI-SPME coupled to GC/EI–MS has showed to be a fast and simple solvent-free method to determine quinalphos in blood and urine samples, and can be regarded as an alternative to the traditional methods, as well as headspace-SPME methods because of high recoveries, low limits and good fibre performance.

The methodology was selective, precise, accurate and sensitive enough for application in forensic toxicology routine analysis for the quantitation of this compound in blood and urine specimens. Furthermore, both techniques require only 100 μL of sample to accomplish the analysis, which is extremely useful when the available sample volume is small.

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