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Determination of organophosphorus pesticides in honeybees after solid-phase microextraction ☆

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Abstract

A method based on solid-phase microextraction (SPME) followed by gas chromatography with nitrogen-phosphorus detection was developed for the purpose of determining 18 organophosphorus pesticide residues in honeybee samples (*Apis mellifera).* The extraction capacities of polyacrylate and poly(dimethylsiloxane) fibers were compared. The main factors affecting the SPME process, such as the absorption time profile, salt, and temperature, were optimized. The method involved honeybee sample homogenization, elution with an acetone:water solution (1:1) and dilution in water prior to fiber extraction. Moreover, the matrix effect on the extraction was evaluated. In samples spiked at the 0.2 mg kg^{-1} level, the coefficient variation was between 1 and 13% and the detection limits were below 10 μg kg^{-1}. The SPME procedure was found to be quicker and more cost-effective than the solvent extraction method commonly used. The method was applied successfully to environmental screening. Parathion methyl was detected and confirmed in the real samples analyzed.

Keywords: Solid-phase microextraction; Organophosphorus compounds; Pesticides

1. Introduction

The use of pesticides in agricultural and plant protection practices could cause extensive pollution of the environment and constitutes a potential risk for human health. These toxic compounds are generally applied over extended areas. Because of this, their presence could be masked by dispersal in the different environmental compartments. However, these compounds tend to become concentrated in certain biological beings named ''bioindicators'', and adequate selection and monitoring of these indicators can help to assess the real impact of pollution. Every day honeybees traverse long distances from their hives to collect nectar and pollen. In the process of doing so, they may be contaminated with surface

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residues of pesticide treatments, which are indicative of the type of environmental pollution [1]. Moreover, bees are highly sensitive to phytosanitary treatments and, depending on the class of product applied, mortality in the colony can also be high [2]. As a result, honeybees have demonstrated to be excellent bioindicators of the pesticides used in a wide agricultural area [3].

Extraction and purification of the target analytes from bees is considered difficult, owing to the large amount of beeswax that is readily extracted by the solvents typically used in residue analysis [4]. Liquid–liquid extraction (LLE) [5], solid-phase extraction on mixtures of silica gel-Florisil [6] or charcoal-silica gel [7], and supercritical extraction [8] followed by GC and LC determination have been suggested for the analysis of pesticides in honeybees. As these extraction procedures present serious drawbacks in that they do not meet the requisites of rapidity, simplicity and economy, easier and faster analytical methods for routine screening of pesticides residues in honeybee samples are needed [3].

Solid-phase microextraction (SPME) is an alternative technique that allows analytes to be directly extracted from an aqueous solution. Unlike other extraction techniques, SPME is not time-consuming, nor does it require large quantities of expensive toxic solvents that are harmful to the environment. In this extraction method the analytes are partitioned between the fused-silica fiber that has been coated with a polymeric stationary phase and the aqueous sample; thus the extraction and concentration steps during sample preparation are joined in a single process. The total analytes retained in the fiber are thermally desorbed in the injector port and deposited at the head of the GC column [8,9]. SPME combined with GC has been successfully applied to the analysis of polar and non-polar analytes including organochlorine [10–13], organophosphorus [14–19], dinitroaniline [14,19] and triazine [12,14,19–21] pesticides.

Up to now, few authors have described the viability of SPME as an extraction technique in complex matrices, such as fruit, vegetables [22,23], honey [24,25] and plants [26]. These products are found mostly in solid, heterogeneous forms, which hinders direct extraction in aqueous samples by SPME. In these cases, the matrix effect should be considered [24].

The aim of this study is to develop an SPME method for determining the pesticide residues in honeybees for future application in environmental control. The utilization of organophosphorus compounds is favored over the more persistent organochlorine and less persistent carbamate pesticides because the former combine two properties, i.e. enough stability to exercise their insecticide action and an ability to degrade in the environment. Therefore, this paper focuses on the SPME of 18 organophosphorus compounds chosen among the most routinely applied pesticides. Attention is focused on the efficiency of different stationary phases and the matrix effects. Moreover, SPME is compared with an LLE procedure used previously in the environmental screening of pesticides [28]. The method developed was applied to real samples of honeybees taken from hives in Emilia Romagna (Italy).

2. Experimental

2.1. Reagents

Pesticide standards (bromophos, chlorpyrifos methyl, coumaphos, diazinon, phenthoate, fonofos, phosalone, phosmet, heptenophos, malathion, methidathion, parathion methyl, parathion ethyl, pyrazophos, pirimiphos methyl, pirimiphos ethyl, quinalphos, triazophos) were provided by Dr Ehrenstorfer, (Augburg, Germany). All pesticide standards were of 98–99% purity. Stock solutions of each organophosphate at 1000 mg l^{-1} were prepared in acetone and stored at 4°C.

They were used to obtain a mixture of 10 mg l^{-1}, from which aqueous solutions for spiking the samples and standard solutions to the required concentration were prepared daily. Acetone and acetonitrile, residue analysis grade, sodium chloride (analysis grade) and Celite were supplied by Carlo Erba Reagenti (Milan, Italy).

2.2. Apparatus and conditions

The SPME holder for use by hand and the manual fiber assemblies were provided by Supelco (Bellefonte, PA, USA). The two SPME fibers were the partially crosslinked 85-μm polyacrylate (PA) phase

and a non-bonded 100-μm polydimethylsiloxane (PDMS) phase. The coated fibers were conditioned according to the manufacturer's instructions to ensure that no contaminants were present. This involved exposing the 85-μm PA fiber in the hot GC injection port at 300°C for at least 2 h, after no peaks were detected; the PDMS fiber was conditioned for 1 h at 290°C. A magnetic stirring unit agitated the sample during SPME.

The GC used was the GC 8000 Series Fisons instrument (Carlo Erba, Milan, Italy) equipped with a nitrogen-phosphorus detection (NPD) system. Separations were obtained using a fused-silica capillary column SPB-608™ (30 m×0.53 mm I.D.) with a 0.5-μm film thickness (Supelco, Bellefonte PA, USA). Desorption of the fibers into the injector port was carried out in the splitless mode at 240°C. The I.D. of the inlet liner for SPME was 0.75 mm. The exposure time of the fiber was 10 min, as this time was considered long enough to ensure the complete desorption of the compounds from the stationary phase and avoid possible memory effects. The optimized temperature program was as follows: initial temperature 60°C increased at 50°C min^{-1} to 120°C, then at 10°C min^{-1} ramp to 250°C and held for 20 min. The carrier gas (He) flow-rate was 15 ml min^{-1}. The detector temperature was 270°C.

To ensure the absence of interference, blank solutions were regularly tested by immersing the SPME fiber in water for 10 min and running a blank injection. In order to avoid the deposition of salt on the GC liner the fiber was washed in clean water for a few seconds after the extraction process and before being inserted in the GC injector.

2.3. Sample description

The honeybee samples used for the SPME study were obtained from environmental monitoring stations located in the Emilia Romagna area (Italy). Preliminary analyses carried out according to a procedure described in a recently published work [28] made it possible to select the bee samples without the studied pesticides.

The samples were lyophilized with a Drywinner Heto1.0-60/CT 60 Cooling Trap (Allerod, Denmark) to eliminate the matrix putrefaction process and derive a better extraction solution.

2.4. Analytical procedure

Three grams of lyophilized honeybees (approximately 10 g of honeybees, fresh weight) were pounded with a glass pestle in a sifter in order to obtain a homogeneous sample. Samples were spiked with 100 μl of a solution containing 10 mg l^{-1} of each pesticide and were allowed to stand at room temperature for 2 h.

The sample was diluted with 50 ml of a solution containing acetone–water (1:1, v/v). Afterwards the solution was shaken vigorously for 30 min and filtered with a Butchner funnel that contained 10 g of Celite over a filter paper. Then, 300 μl of the filtrate was diluted in 3 ml of water solution and placed in a 4-ml sample vial. The final concentration in acetone was 5%. Optimum mixing of the water solution was achieved by magnetic stirring. Solutions were stirred at a regular speed of 700 rpm; a higher speed in the manual procedure was not practicable because the parabolic void formed by the magnetic stirring forced an immersion of the fiber near the walls of the vial. It has been observed that the stirring process may produce air bubbles in the stationary phase and this could have a significant effect on precision. The solution should be degasified by sonicating the vial prior to the extraction process.

2.5. Quantification

Quantification was performed using the spiked samples: 1 μg of each pesticide was added to the honeybee matrix (100 μl of a solution containing 10 μg ml^{-1} of the pesticide mixture) and diluted to 50 ml; from this solution an aliquot of 300 μg was further diluted to 3 ml. Therefore the stock solution was diluted 500 times (1 μg/10×50 ml) and the final concentration was 0.002 μg ml^{-1} in 5% aqueous acetone. The standard solution was prepared in order to obtain the same final concentration as well as the same percentage of acetone. From the stock solutions of each organophosphorus at 1000 mg l^{-1} a solution was obtained with a concentration of 0.04 μg ml^{-1} of each pesticide in acetone. An aliquot of 150 μl was diluted to 3 ml of water to obtain 0.002 μg ml^{-1} in 5% acetone. The same calculations were performed to arrive at the different concentrations used in the linearity studies.

3. Results and discussion

3.1. Optimization of the SPME

The different parameters that can affect the SPME process (the selected phase, immersion time, extraction temperature and ionic strength) were optimized by analyzing vials containing 2 μg l^{-1} of target compounds and 5% of acetone.

3.1.1. Comparison of fiber coating

Both fibers, 85-μm PA phase and 100-μm PDMS, were immersed for time intervals increasing from 5 to 120 min. The time required for the analytes to reach equilibrium between the aqueous and the stationary phase was less than 120 min in the case of the PDMS fiber, whereas equilibrium was not reached even after 120 min when the PA fiber was used [14]. PDMS fiber has the property of a liquid whereas PA is a solid [17] and consequently faster diffusion and a shorter equilibrium time may be expected when PDMS is used [8,17].

The amount of each analyte extracted by the fibers was plotted against the extraction time and the graphs were divided into three models according to the shape of the curve. Fig. 1 shows the equilibrium time profile of pyrazophos, phenthoate and phosmet between the aqueous phase and the two fibers. These compounds are representative of each of the three different behaviors observed. Pyrazophos, parathion ethyl, parathion methyl, methidathion, quinalphos, triazophos, coumaphos, phosalone and malathion constitute the first group, where the amount extracted with PDMS was significantly lower than with PA fiber and equilibrium was reached in 30–50 min using PDMS fiber. The second group is made up of phenthoate, bromophos, chlorpyrifos methyl, diazinon, heptenophos, fonofos, pirimiphos methyl and pirimiphos ethyl and showed a comparable extraction with both fibers. Phosmet differs from the other compounds in that it was not extracted at the studied concentrations with the PDMS fiber.

Fig. 2 compares the gas chromatograms of the 18 organophosphorus pesticides extracted by PA (Fig. 2A) and PDMS (Fig. 2B). From these figures it may be seen that the extracted amount of target analytes was greater with PA than with PDMS, because of the stronger affinity of polar analytes such as organophosphorus compounds for PA fiber. Moreover, compared with the PDMS phase, PA fiber tolerates much higher concentrations of organic solvents. Therefore, optimal extraction was achieved with 85-μm PA.

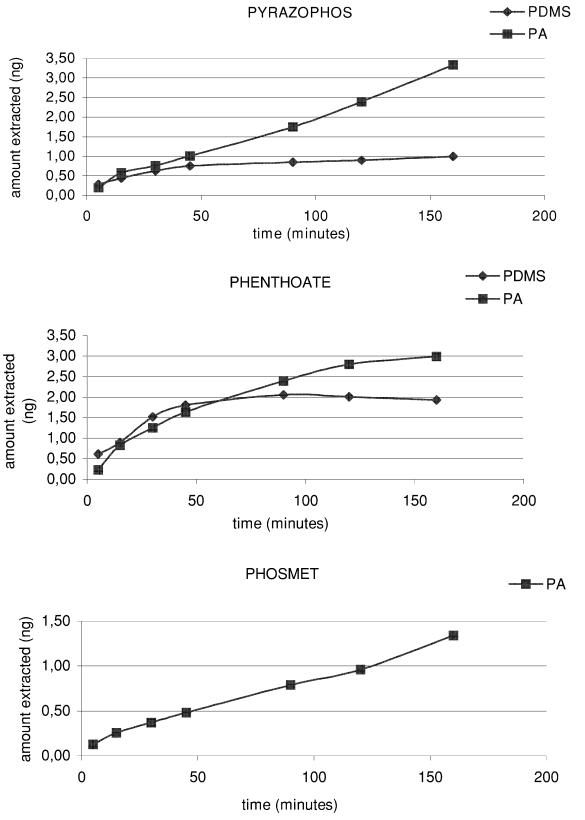

Fig. 1. Equilibrium time profile of three organophosphorus pesticides with the 85-μm polyacrylate (PA)- and 100-μm poly(dimethylsiloxane) (PDMS)-coated fiber.

Since SPME is a process dependent on equilibrium rather than total extraction, a shorter equilibrium time can be attained. An immersion time of 45 min was selected because the response that may be obtained in this period of time is acceptable and it is thus appropriate for routine analysis [18]. Precision and sensitivity may be affected, mainly in manual operation; for this reason the exposure time of the fiber should be carefully controlled [12].

3.1.2. Temperature effect

In order to study the effect of temperature on the extraction process, vials were immersed in a water

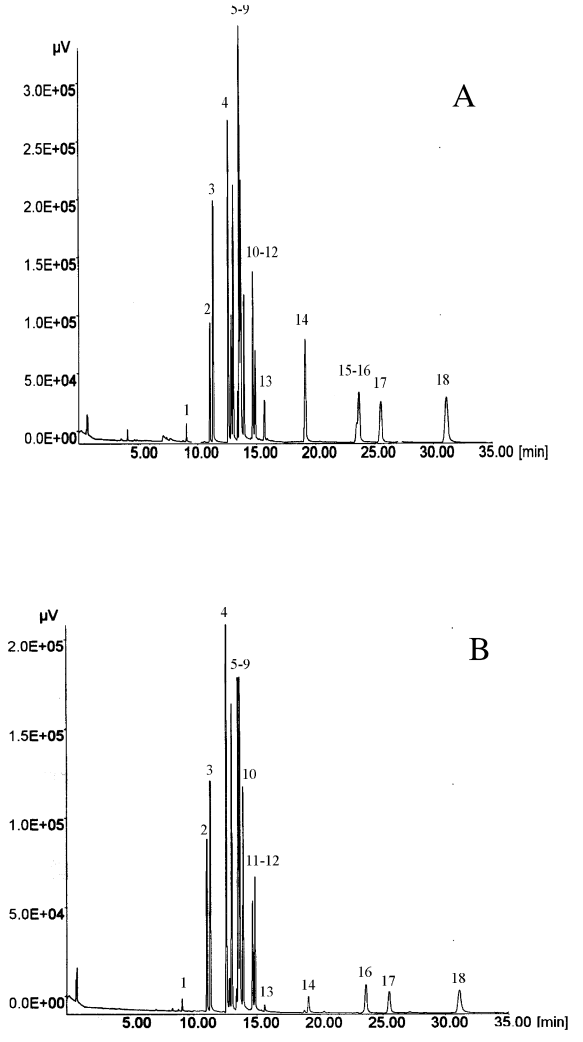

Fig. 2. Chromatograms of 18 organophosphorus pesticides after extraction of an aqueous solution, spiked at 2 ng ml^{-1} with two fibers: (A) 85-μm polyacrylate and (B) 100-μm poly(dimethylsiloxane). 1=heptenophos, 2=diazinon, 3=fonofos, 4=chlorpyrifos methyl, 5=parathion methyl, 6=pirimiphos methyl, 7=parathion ethyl, 8=malathion, 9=parathion methyl, 10=bromophos, 11=quinalphos, 12=phenthoate, 13=methidathion, 14=triazophos, 15=phosmet, 16=phosalone, 17=pyrazophos, 18=coumaphos.

bath heated by the magnetic stirring unit. A thermometer was used to monitor the water temperature.

The temperature effect was evaluated by varying the temperature from 25 to 60°C. An increase in extraction temperature caused an increase in the extraction rate and a simultaneous decrease in the distribution constant between the analytes and the fiber [8]. As is shown in Table 1, the effects of this parameter varied considerably among the different pesticides. The response of parathion ethyl, bromophos and pirimiphos ethyl increased at 60°C whereas at lower extraction temperatures, heptenophos, phenthoate and methidathion showed stronger responses. The analysis of the 18 organophosphorus compounds was performed without considering the effect of temperature. The magnetic stirring may generate heat during the stirring operation, affecting the reproducibility of the measurement [8]. To maintain a constant temperature vials were raised a few millimeters from the base.

Table 1
Comparison in the amount (ng) extracted from a mixture with 2 μg ml^{-1} of each pesticide using different extraction temperature by the polyacrylate fiber (n=3)

Pesticide	Amount extracted (ng)			
	25°C	40°C	50°C	60°C
Bromophos	0.56	0.57	0.63	0.67
Chlorpyrifos methyl	0.79	0.65	0.71	0.89
Coumaphos	1.24	1.25	1.33	1.44
Diazinon	0.32	0.24	0.29	0.30
Fonofos	0.42	0.40	0.46	0.46
Heptenophos	0.06	0.05	0.04	0.03
Malathion	0.23	0.17	0.14	0.15
Methidathion	0.21	0.13	0.11	0.07
Parathion ethyl	1.03	1.01	1.08	1.18
Parathion methyl	0.24	0.13	0.11	0.13
Phenthoate	0.65	0.50	0.39	0.29
Phosalone	0.48	0.41	0.40	0.42
Phosmet	0.11	0.05	0.06	0.06
Pirimiphos ethyl	0.71	0.75	0.78	0.92
Pirimiphos methyl	1.01	0.91	0.97	1.07
Pyrazophos	0.55	0.54	0.65	0.74
Quinalphos	0.67	0.50	0.57	0.46
Triazophos	1.06	0.89	0.80	0.83

3.1.3. Salt effect

The effect of ionic strength on extraction efficiency was evaluated by analyzing the amount of pesticides extracted in sample solutions containing 10, 20, and 30% (w/v) of sodium chloride. Pesticides that are more soluble in water have a lower affinity for the fiber coating. The amount of these analytes extracted by the fiber can be increased if the solubility of the analytes in water is decreased by adding sodium chloride to alter the ionic strength

[11]. The response of heptenophos and malathion increased in proportion to the amount of sodium chloride, but chlorpyrifos methyl, coumaphos and triazophos showed the opposite effect. The values of the octanol–water partitioning coefficient ($\log P$) ranged from 2.32 of heptenophos to 5.07 of bromophos methyl. Given this wide range of hydrophobicities and these opposite behaviors no salt modifications were considered.

3.1.4. Influence of organic solvents

It is conceivable that a high organic solvent content precludes an efficient extraction [14]. Samples of standard solution at 2 ng ml^{-1} without organic solvent, with 5 and 15% of acetone and 5 and 15% of acetonitrile, were studied. There seems to be no noteworthy difference between the two solvents. The 5% (v/v) acetone in an aqueous solution only reduces the peak response by about 3–10% compared with the peak response of the standard solution at the same concentration in water. The same behavior has been observed by other authors with other organic solvents such as methanol [14]. Therefore, acceptable extraction may still be achieved at this concentration of organic solvent. Acetone was selected because it is non-toxic, easy to volatilize and low-cost.

3.2. Matrix effects

SPME is an equilibrium method and not an exhaustive method such as LLE or SPE, whose primary aim is to obtain a quantitative extraction of the analytes in the extraction phase. As a consequence, with the latter methods selectivity is often sacrificed because many matrix components are co-extracted. Equilibrium methods such as SPME, in which analytes are absorbed into the fiber directly from an aqueous sample, are more selective because they take full advantage of the differences in the partition process to separate target analytes from interference [27].

The extraction procedure for the analysis of pesticides in honeybees was studied by diluting the sample with 50 ml of water and shaking it prior to PA fiber immersion in a 3-ml aliquot. In order to assess the matrix effect on peak response, recovery was calculated by comparing the area of each peak obtained from the extraction of the standard in an aqueous solution with the area of the peaks obtained from honeybee samples spiked at the same concentration and extracted using the same procedure. The recoveries obtained were very small, apparently because removing pesticides from the honeybee matrix with water only is not very effective, especially as regards the more lipophilic pesticides. Hence the water dissolved a large amount of organic matter and this suspended matter interfered in the extraction process [17].

In attempt to reduce the matrix effect and to ameliorate analyte recovery, the honeybee matrix was diluted with 25, 50 and 75% of an acetone–water solution and different aliquots were taken in order to obtain a final extract of 5% of acetone. As the results shown in Fig. 3 indicate, the peak response obtained using a solution of acetone–water (1:1) was more comparable to the standard response in an aqueous solution than the other proportions used. With this proportion, the dissolved polar organic matter diminished and the resulting extract was cleaner. A high acetone content dilutes the beeswax that interferes with pesticide extraction and a stronger dilution is needed to obtain a final extract of 5% acetone.

The matrix effect may be assessed for the majority of the pesticides studied on the basis of the data provided in Table 2, which shows the recovery obtained by triplicate analysis of honeybee samples spiked at 0.2, 0.1, 0.05 and 0.01 mg kg^{-1}. As can be

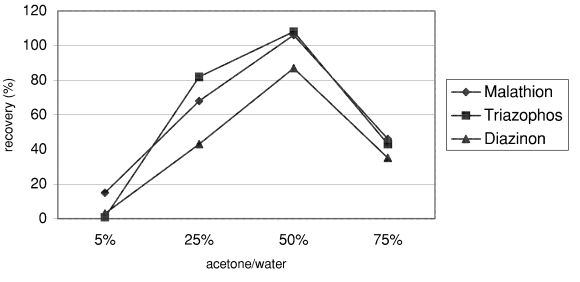

Fig. 3. Recovery obtained after extracting a honeybee sample spiked at 0.1 mg kg^{-1} and diluted with various proportions of water–acetone.

Table 2
Recoveries and CV values of pesticides extracted from honeybees fortified at various concentrations ($n=3$)

	Pesticide	0.2 mg kg^{-1}		0.1 mg kg^{-1}		0.05 mg kg^{-1}		0.01 mg kg^{-1}	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
1	Heptenophos	86	9	106	6	123	6	92	2
2	Diazinon	106	8	107	4	114	3	96	2
3	Fonofos	101	7	87	4	97	2	84	3
4	Chlorpyrifos	79	11	77	6	90	2	69	3
5	Parathion methyl	107	7	116	5	118	3	121	4
6	Pirimiphos methyl	100	6	96	4	99	3	77	5
7	Malathion	103	1	116	7	131	5	97	5
8	Parathion ethyl	134	6	104	4	124	3	95	1
9	Pirimiphos ethyl	77	8	56	6	77	7	45	10
10	Bromophos	49	13	38	8	56	2	46	3
11	Quinalphos	117	4	110	4	116	1	86	7
12	Phenthoate	90	5	122	5	118	9	96	2
13	Methidathion	154	8	203	5	201	12	250	30
14	Triazophos	121	4	128	5	128	1	124	2
15	Phosmet	109	6	90	1	73	12	90	3
16	Phosalone	94	7	92	4	114	4	100	3
17	Pyrazophos	103	5	101	6	125	6	94	4
18	Coumaphos	96	8	95	5	113	5	98	3

observed, more lipophilic compounds such as bromophos and pirimiphos ethyl had a negative matrix effect. There was an inverse effect in the case of triazophos and parathion methyl, where the matrix effect increased the extraction efficiency. Methidathion had a percentage above 200% because at the same retention time there was an interference peak from the extract. Different blank injections were performed and an interference peak was observed in the same retention time as methidathion, which should explain a CV of 30%.

Besides the lipophilic character of the pesticides and the ionic strength of the matrix, other unknown parameters inherent in the nature of the matrix itself can also influence pesticide extraction and must be taken into consideration.

Table 2 summarizes the CV values of the honeybee samples spiked at four levels. Precision ranged from 1 to 13% except in the case of methidathion, which gave an CV of 30% due to the problems reported above. The regression coefficients and detection limits are shown in Table 3: they ranged from 0.9619 to 0.9996. The detection limits ranged from 10 μg kg^{-1} for heptenophos, diazinon, malathion and phenthoate to 1 μg kg^{-1} for chlorpyrifos.

Table 3
Analysis of spiked samples of honeybees, limits of detection and coefficient of regression

Pesticides	Coefficient of regression (R^2)	Limits of detection (μg kg^{-1})
Heptenophos	0.9993	10
Diazinon	0.9969	10
Fonofos	0.9878	7
Chlorpyrifos	0.9704	1
Parathion methyl	0.9904	8
Pirimiphos methyl	0.9881	2
Malathion	0.9958	10
Parathion ethyl	0.9619	9
Pirimiphos ethyl	0.9996	3
Bromophos	0.9971	9
Quinalphos	0.9919	5
Phenthoate	0.9794	10
Methidathion	0.9851	10
Triazophos	0.9953	5
Phosmet	0.9951	10
Phosalone	0.9909	10
Pyrazophos	0.9962	3
Coumaphos	0.9930	4

3.3. Application to real samples

The method was optimized using spiked samples of honeybees; six real samples were extracted using SPME. Fig. 4 shows, by way of example, the chromatogram of a positive honeybee sample analyzed for the environmental screening of pesticides in the Bologna area during 1998–1999 using SPME (Fig. 4A) and LLE (Fig. 4B), which led to the detection of parathion methyl at 1.5 and 1.2 mg kg^{-1}.

The samples used for the recovery test were taken from the negative honeybee samples analyzed in a monitoring program conducted in Bologna where an LLE procedure using acetone and dichloromethane with a coagulant solution to eliminate wax content was successfully applied [28].

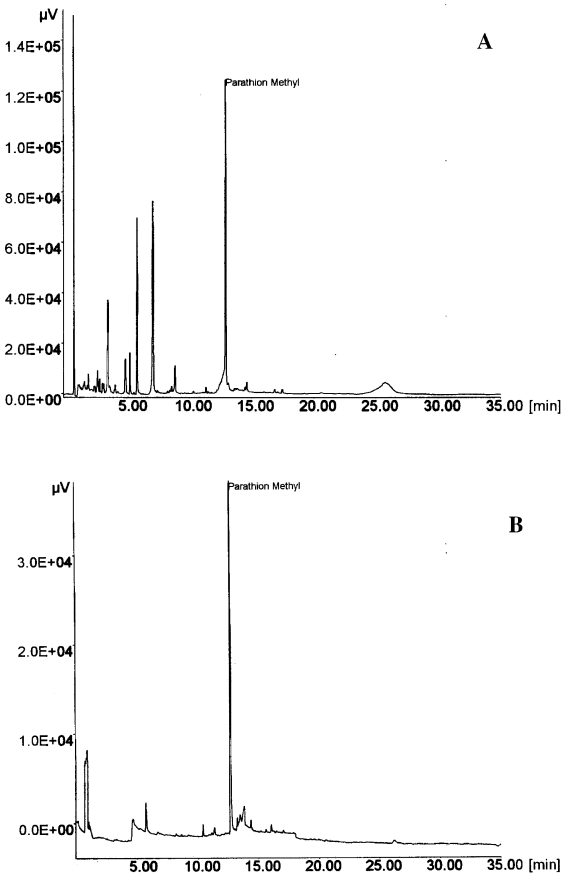

Fig. 4. Chromatograms of honeybee sample containing parathion methyl, derived using: (A) liquid–liquid extraction and (B) solid-phase microextraction.

As a result of the selectivity of polymeric fiber for the compounds studied, the chromatogram obtained with SPME shows less interference than the LLE-derived chromatogram.

3.4. Conclusion

SPME has demonstrated to be a fast, simple, solvent-free method for extracting pesticides from water samples. A simple solution in water does not seem to be useful in the case of a complex matrix such as that of honeybees. Taking into account the high sensitivity of the SPME technique and the effect of matrix interference on the extraction process, sample dilution and the use of organic solvents have proven to improve the extraction efficacy of the SPME technique with biological samples.

Although many aspects of the application of SPME for analyzing pesticide residues in complex samples still have to be investigated, this extraction technique may be considered a viable alternative to multiresidue techniques. The convenience, simplicity and reliability of the SPME technique should undoubtedly make it a valuable tool for the environmental screening of pesticides in the future.

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