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Solid phase microextraction gas chromatographic analysis of organophosphorus pesticides in biological samples

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Abstract

Headspace-solid phase microextraction (HS-SPME) was studied and optimised for the determination of four common organophosphorus pesticides (OPPs) in biological samples. Various parameters controlling SPME were studied: choice of SPME fiber, type and content of salt added, preheating and extraction time, desorption time, extraction temperature. Capillary gas chromatographic analysis with nitrogen phosphorus detection (GC–NPD) facilitates sensitive and selective detection of the OPPs: malathion, parathion, methyl parathion and diazinon. Fenitrothion was used as the internal standard. The method was applied to the determination of the pesticides in human biological specimens: whole blood, blood plasma, urine, cerebrospinal fluid, liver and kidney. Limits of detection ranged from 2 to 55 ng/ml depending on pesticide and type of specimen. The developed methodology overcomes limitations and obstacles of conventional methods such as the use of organic solvents, the formation of emulsions and the tedious-cumbersome procedures. The proposed protocol is seen as an attractive alternative to be used in routine toxicological analysis.

Keywords: SPME; Organophosphorus pesticides; Biological samples; Sample preparation

1. Introduction

Organophosphorus pesticides (OPPs) are among the most commonly employed pesticides world-wide. On a global basis, intoxications attributed to these pesticides have been estimated to be as high as 3 million cases of acute and severe poisoning annually, with as many or more unreported cases and with some 220,000 deaths. The easy availability in agricultural areas, their high toxicity and the rapid action of these compounds are the main reasons for their use in suicide cases [1].

Organophosphorous pesticides are very toxic when absorbed by human organisms because of acetyl-cholinesterase de-activation. Symptoms of acute organophosphate poisoning develop during or after exposure, within minutes or hours, depending on the method of contact. Exposure by inhalation results in the fastest appearance of toxic symptoms, followed by ingestion and finally the dermal route. Acute toxicity results in acute pneumonary edema and death.

Several methodologies have been developed for sample preparation prior to the analysis of pesticides from biological or environmental samples [2,3]. In many cases methods employ complex extraction protocols that can be time and solvent consuming, cumbersome and prone to experimental errors. An attractive alternative introduced recently in sample preparation is solid phase microextraction (SPME) [4–10]. This technique integrates sampling, extraction, preconcentration and sample introduction in a simple single step procedure. Although SPME was initially introduced for the extraction of organic compounds from aqueous environmental samples [4], the method has gained interest in a broad analytical field including food, biological and pharmaceutical analysis [8–10]. Furthermore, the development of headspace-solid phase microextraction (HS-SPME) [5] provided a powerful alternative for the sampling and

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pre-treatment of various biological samples such as urine, whole blood, plasma and hair. Direct immersion and in-tube SPME protocols have also been used, but they may result in shortened fiber lifetime or capillary clogging [7–10].

SPME has been successfully applied for the determination of pesticides in various samples [11–19] as comprehensively reviewed by Beltran et al. [11]. The exploitation of SPME for the treatment of samples of biological origin is limited, especially when compared to environmental or food analysis. Headspace sampling is generally preferred for blood or urine sampling [11–15,19]. Methodical optimisation for the HS-SPME of OPPs in whole blood has been reported by Mushoff et al. [15] and Hernandez et al. [12]. The selection of the biological specimen to be analysed is crucial. Although whole blood is a more complex sample compared to plasma and serum, in post-mortem toxicological analysis whole blood has to be assayed, because plasma or serum cannot be obtained from blood. Furthermore, it is often of interest to obtain information on the levels of certain compounds before their distribution in different blood compartments. In fatal intoxications, the distribution of the toxic compound in different tissues is of utmost importance. In certain cases where the diagnosis of overdose is to be used in judicial evidence, a single sample of blood may prove insufficient. In such cases, analyses of several samples of blood and tissue will increase the possibility of reaching a correct conclusion as reference values of pesticide concentrations in tissues are missing [20]. OPPs are metabolized (enzymatically hydrolysed) in liver and in plasma. Most of studies of control exposure are directed towards the determination of OPP metabolites in blood or urine. The parent pesticides can be normally expected in cases of acute intoxications or poisoning, where the concentration levels of the pesticides are high enough to be detected unaltered in biological fluids or tissues, in spite of their rapid metabolism. Sensitive and selective methods are necessary for the analysis of OPPs in whole blood and human/animal tissue. Hence the development of practical and efficient sample preparation methods for the determination of the OPPs in biological fluids and tissue (liver, kidney) is of great interest.

Recently we have reported on the determination of methyl parathion in post-mortem tissue using HS-SPME method in combination with gas chromatography (GC) with a nitrogen–phosphorous detection (NPD) [19]. In the present paper, the scope of the work was extended to the analysis of various tissues for the determination of OPPs: parathion, methyl parathion, malathion and diazinon. The experimental methodology used was again based on HS-SPME and GC–NPD and was developed following a systematic study of essential factors influencing analysis and extraction. SPME was optimized by studying the effect of several experimental parameters: extraction temperature, salt addition, sample pH value, preheating and extraction time. The selected scheme was applied to the determination of the OPPs in human biological samples: whole blood, blood plasma, cerebrospinal fluid, liver and kidney tissue.

2. Materials and methods

2.1. Reagents

All reagents were of analytical or higher grade and were purchased from Merck (Darmstadt, Germany). Solvents were of HPLC grade and were from Merck. Methyl parathion, malathion, parathion, fenitrothrion and diazinon were purchased in the form of certified material from Dr. Ehrenstorfer Reference Materials (Augsburg, Germany). The molecular structures of the analysed pesticides are given in Fig. 1. Fenitrothion is a pesticide still in use and may be present in certain real samples. In such a case another of the studied pesticides should be selected as the internal standard.

2.2. Gas chromatography

Gas chromatographic analysis was performed using a Thermo Finnigan Trace GC–NPD (Thermoquest Italia,

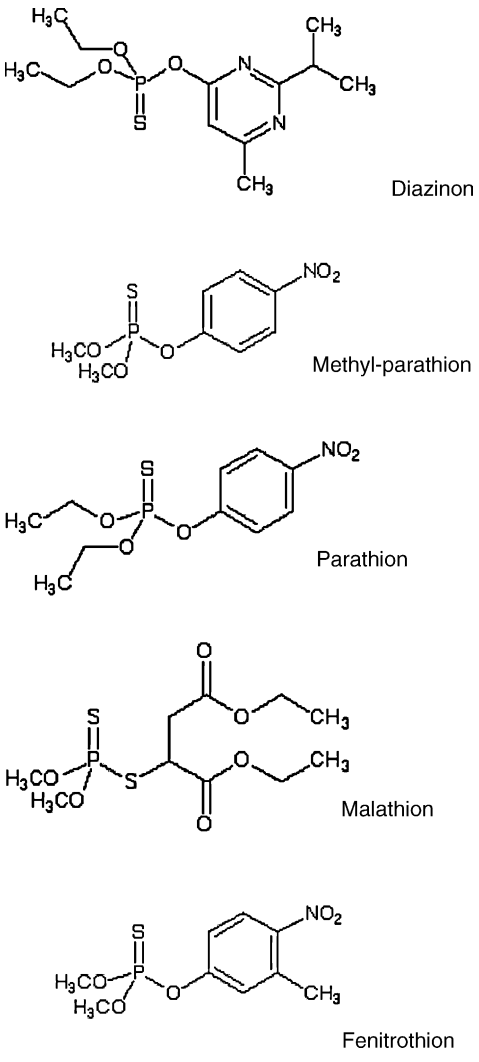

Fig. 1. Molecular structures of the analysed pesticides.

Rodano, Italy). Separations were accomplished on an Alltech EC-5 (30 m × 0.32 mm, 0.25 μm) column (Alltech, Deerfield, IL). The oven temperature was held at 120 °C for 3 min and then increased to 230 °C at a rate of 10 °C/min, where the temperature was held for 4 min. The temperatures of the injector port and the detector were set at 230 and 300 °C, respectively. Splitless injection mode was used. Helium was used as the carrier gas at a flow rate of 2 ml/min.

2.3. Development of SPME procedure

SPME holder and fibers were obtained from Supelco (Bellefonte, PA, USA). Polyacrylate (PA, 85 μm) and polydimethylsiloxane (PDMS, 100 μm) fibers were used. For SPME development selected volumes of a methanolic solution (50 μg/ml) of the OPPs and 100 μl of a internal standard solution (60 μg/ml solution of fenitrothion methanolic solution) were transferred to a headspace vial (9 ml volume from Alltech, Alltech Ass. Deerfield, IL) where they were evaporated to dryness under a gentle stream of N_2. Next a certain amount of salt was added. The sample was reconstituted to solution with the addition of 3 ml of Millipore water and the vial was next capped tightly, shaken thoroughly in a vortex machine and was placed for thermostating in an aluminum block heater. After 15 min (preheating period), the SPME needle pierced the vial septum in order to expose the fiber in the headspace of the solution. Sampling was performed for a certain time and finally the needle was removed from the vial and was inserted into the heated injection port of the gas chromatograph for desorption for 4 min. In certain experiments in order to study the influence of the sample pH, extraction was performed following acidification with appropriate volumes of a 2 M H_2SO_4 solution.

2.4. Processing of biological samples

Drug and pesticide free blood samples to be used in study were collected from healthy adults. Similarly to the SPME procedure described above for the reference solutions, 100 μl of a methanolic solution of I.S. (60 μg/ml) were placed in a headspace vial and were next evaporated to dryness. 0.8 g NaCl was added and next 300 μL of the plasma sample was transferred to the vial. 2.7 ml of Millipore water was added and the vial was closed and thoroughly shaken in a vortex shakes for 5 min. Next the sample was processed with SPME under the selected conditions.

Post-mortem biological samples (liver, kidney, cerebrospinal fluid and whole blood) free of drugs and pesticides were collected from autopsy cases. Prior to SPME, liver and kidney tissues were treated as follows: 1 g of tissue was homogenized along with two ml of water using a high speed blender. Three hundred microliters of the tissue homogenate or biological fluid were transferred to the SPME vial. Using distilled water, the total volume of each solution was adjusted to 3 ml. The resulting solution was subjected to extraction as described above.

To produce spiked biological samples appropriate volumes of methanolic solutions of OPPs (500 μg/ml for high concentration and 50 μg/ml for low concentration samples) and a constant volume of a methanolic internal standard solution (100 μl of a 60 μg/ml solution) were placed into a headspace vial. The solution was evaporated by a gentle stream of N_2. Then 0.8 g of NaCl were placed into the vial. Subsequently 300 μl of the corresponding biological sample and 2.7 ml of distilled water were added. The vial was next capped tightly and shaken thoroughly, in order to be homogenized and the resulting solution was subjected to SPME as described above.

3. Results and discussion

3.1. SPME optimisation

The first aim was the development of an efficient, accurate and precise GC method for the simultaneous determination of the four OPPs and the internal standard in biological samples. The developed GC protocol provided satisfactory separation power and a baseline resolution of the five analytes within 12 min. The utilisation of GC–MS as the analytical tool would be preferable, due to the additional useful information the method can provide (including confirmation and metabolism investigation capabilities). However, since such a system was not available in the laboratory, the GC–NPD combination provided a very efficient tool with very satisfactory sensitivity and selectivity: clear chromatograms were obtained even for extracts from complex biological materials (see Section 3.2).

Direct immersion SPME has been applied by other researchers to the analysis of OPPs in environmental samples and blood plasma [11,17,18], however, in the majority of the reported works, the HS mode is preferred. The purpose of our study was the development of an experimental protocol that should address the processing of a large number of real samples including very complex biological tissue such as liver and kidney. Therefore, the HS mode was preferred, which in fact facilitated satisfactory analytical performance and longer fiber life time.

Next SPME was studied in detail on spiked water samples (2 μg/ml level for each selected OPPs and IS), each sample being assayed thrice. It should be pointed that although almost all the researchers follow similar SPME optimization procedures, critical differences occur in the selected experimental conditions reported in the literature. A systematic optimisation of the SPME experimental procedure was thus deemed necessary to achieve higher yields and enhance method sensitivity. Parameters including fibre type, temperature, salt addition sample pH, extraction and desorption time were step-by-step studied and optimized.

The first parameter studied was the type of fiber. Preliminary experiments revealed much higher signals when using the PA fiber compared to the PDMS fiber. Hence the PA fiber was selected for the study.

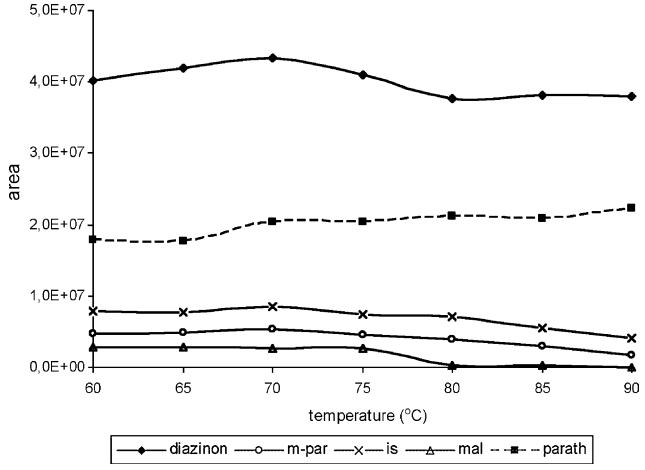

Fig. 2. Plot of peak area ratio of the analytes vs. the extraction temperature. *Abbreviations*: mal, malathion; parath, parathion and m-par, methyl parathion; IS: fenitrothion. *Conditions*: PA fiber, 0.4 g NaCl added, 20 min extraction time. *Concentrations*: diazinon, malathion, parathion, methyl parathion and IS: 2 μg/ml.

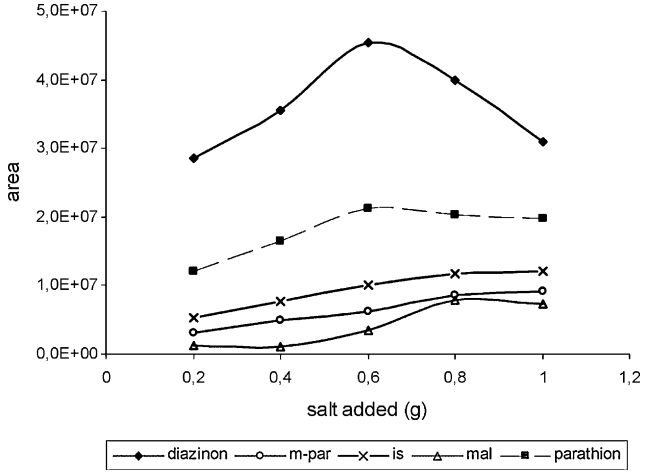

Fig. 3. Plot of peak areas of the analytes vs. the mass of salt (NaCl) added in the sample prior to SPME. *Abbreviations*: mal, malathion; parath, parathion and m-par, methyl parathion; IS: fenitrothion. *Conditions*: PA fiber, 20 min extraction time, 70 °C. *Concentrations*: diazinon, malathion, parathion, methyl parathion and IS: 2 μg/ml.

Temperature is a fundamental parameter for HS-SPME. In general an increase in temperature can increase the extraction yield in non-equilibrium situations, but may also decrease the distribution constant into the fiber. In order to determine the effect of temperature on the absorbed amount of analyte, the samples were heated for 20 min at a series of increasing temperatures. Only for parathion higher sensitivities were actually observed reached at elevated temperatures. For the rest three pesticides a somewhat lower detector signal was obtained in temperatures higher than 70 °C (Fig. 2). This could also be attributed to analyte instability as malathion and diazinon are reported to decompose at excessive temperatures. Therefore, a moderate temperature (70 °C) was chosen for the further experiments.

Salt addition can improve the extraction yield in SPME as a result of a "salting out" effect. Salts often employed include NaCl, $(NH_4)_2SO_4$, Na_2CO_3 in varying contents. The salts studied in the present work were: NaCl, $(NH_4)_2SO_4$ and NH_4Cl. To select the optimum salt for this study, extractions were performed following the addition of an amount of 0.4 g of the above mentioned salts. The recovery obtained for OPPs in the presence of NaCl was higher than those of $(NH_4)_2SO_4$ and NH_4Cl. A more detailed study was then conducted concerning the amount of NaCl salt added (0.2–1 g added in the sample). Fig. 3 illustrates the results obtained from this study. Typically an increase in the amount of salt results to increasing extraction yields up to a maximum which is followed by a decrease with further saline increment. In this case it is believed that polar analytes contributing to electrostatic interactions in saline environment, lose their mobility and mass transfer towards the extracting phase. This phenomenon was observed in this study for parathion and diazinon. As seen in Fig. 3, a decrease is observed in the extraction yield of these two pesticides for amounts higher than 0.6 g. In contrast, for the rest two analytes and the IS a constant increasing trend is observed. Enhancement of the detection signal of malathion and methylparathion was considered more crucial as these pesticides provided lower signals. Therefore, the amount of 0.8 g of NaCl was selected for the rest of the study.

Adjustment of pH may improve the extraction yield for compounds that can be protonated. In most of the cases pH is adjusted in order to obtain the analyte in its neutral undissociated form to enhance extraction yield, because only this form is extracted in the absorptive fiber. Addition of sulfuric acid (50 μl of a 2 M solution) did not improve the extraction yields of the pesticides (except for a slight improvement in the recovery of parathion). In contrast acidification resulted in reduced signals for diazinon (see Fig. 4) probably due to the protonation of the two pyrimidine N atoms. Therefore, acidification of the sample was omitted.

Finally, the length of preheating and sorption steps was studied. SPME may function as an equilibrium technique or

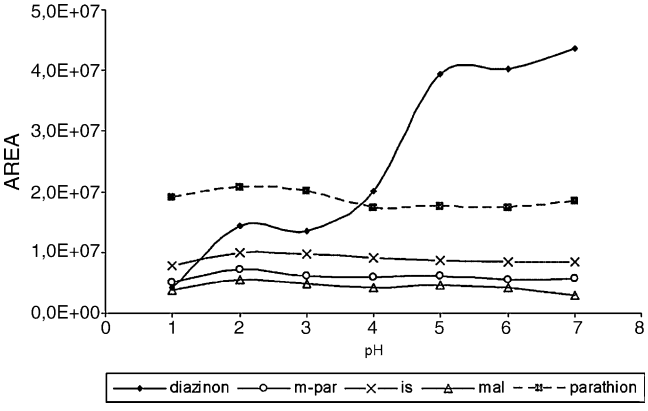

Fig. 4. Plot of peak areas of the analytes vs. pH value of the solution. *Abbreviations*: mal, malathion; parath, parathion and m-par, methyl parathion; IS: fenitrothion. *Conditions*: PA fiber, 20 min extraction time, 70 °C. *Concentrations*: diazinon, malathion, parathion, methyl parathion and IS: 2 μg/ml.

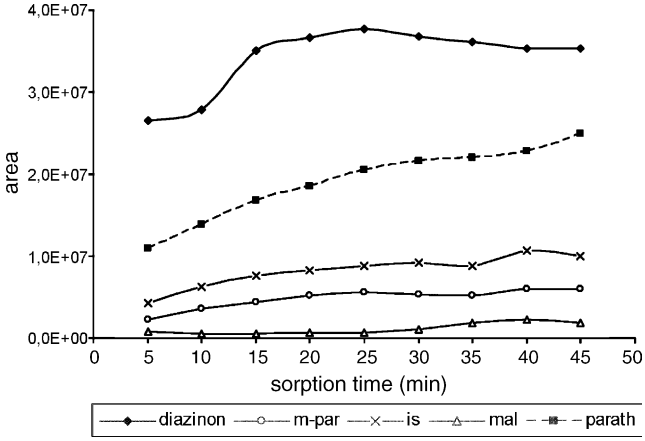

Fig. 5. Plot of peak areas of the analytes vs. the extraction (sorption) time. *Abbreviations*: mal, malathion; parath, parathion and m-par, methyl parathion; IS: fenitrothion. *Conditions*: PA fiber, 0.8 g NaCl added, temperature 70 °C. Concentrations for all pesticides and IS: 2 μg/ml.

as a sampling technique for a selected time. The preheating step was used in order to prepare a sample for SPME. The vial was placed in the aluminum bloc heater for 15, 20, 30 and 40 min. Next the SPME fiber was inserted to the vial for extraction. The variation of preheating time did not affect the extraction recovery, thus the shortest time of 15 min was selected for the rest of the study. Next, extraction time varied from 5 to 45 min in order to obtain a saturation curve. Samples were assayed with the selected conditions (addition of 0.8 g NaCl and heating at 70 °C). Fig. 5 shows the influence of the variation of the extraction times on the analyte signal. Only for parathion longer sorption times did result in signal enhancement. Therefore, a fixed time of 20 min was finally chosen as it provided satisfactory recovery for the pesticides and for the sake of overall analytical time.

An exposure time of four minutes in the GC injector port, was found sufficient for optimum recovery and complete analyte desorption. Effective fiber clean-up in the injector port eliminated carry-over phenomena between subsequent analyses.

3.2. Analysis of biological samples—validation

Following optimization of the HS-SPME for the selected OPPs, the next step was the application of the method in their simultaneous determination in biological samples: liver, kidney, cerebrospinal fluid, whole blood and plasma. Typical chromatograms obtained from the analysis of biological samples are in depicted in Figs. 6 and 7. Fig. 6 shows the chromatogram obtained when applying the developed SPME–GC procedure to human whole blood samples. Fig. 6A shows the chromatogram of blank blood, Fig. 6B the analysis of the same sample following spiking at 50 ng/ml for each OPP and Fig. 6C the analysis of a post-mortem blood sample obtained from a human subject following intravenous injection of malathion (for more details, see ref. [19]). Fig. 7 shows the chromatogram obtained when applying the developed

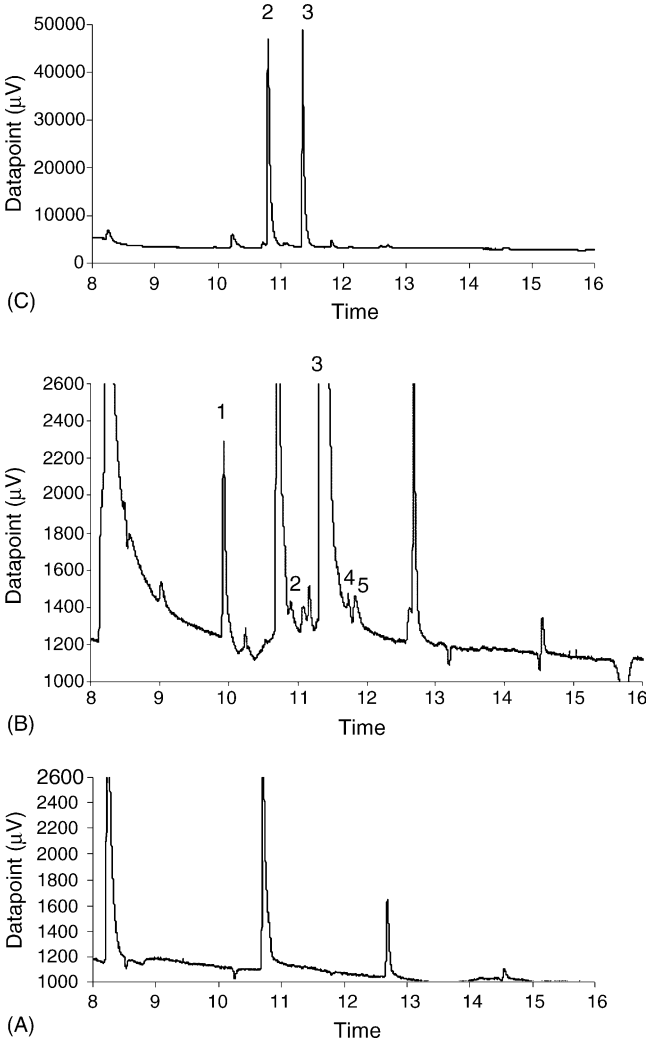

Fig. 6. GC–NPD chromatogram following HS-SPME from human whole blood sample. (A) Blank blood sample, (B) the same blood sample following spiking with the studied OPPs (50 ng/ml for the OPPs, 2 μg/ml for the IS). (C) Post-mortem blood obtained from a human subject following administration of malathion Peak identities: 1, diazinon; 2, methyl-parathion; 3, internal standard; 4, malathion; 5, parathion.

SPME procedure to the analysis of blank kidney (Fig. 7A) and spiked kidney (Fig. 7B) (100 ng/ml for the OPPs). In all cases sharp peaks and good chromatographic resolution for each compound was obtained. As seen also in the analysis of blank samples clear chromatograms were acquired with no interferences from the matrix.

In order to construct calibration curves, biological samples spiked with the selected OPPs at final concentrations ranging from 0.02 to 20 μg/ml (or μg/g) were prepared and analyzed with the described procedure. Each calibration level was analysed in triplicate. The calibration curves were obtained by plotting the peak-area ratio between the analytes and fenitrothion (internal standard). Regression analysis was used to construct calibration curves. Diazinon was found to be linear in the tested range with correlation coefficients (R^2) from 0.9866 to 0.9999. For parathion, the calibration

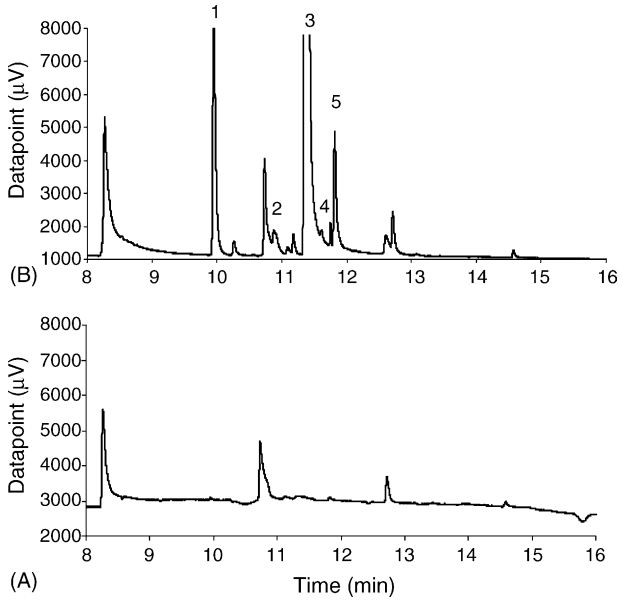

Fig. 7. GC–NPD chromatogram following HS-SPME from homogenised human kidney tissue. (A) Blank kidney sample, (B) the same kidney sample following spiking with the studied OPPs (100 ng/ml for the OPPs, 2 μg/ml for the IS). Peak identities: 1, diazinon; 2, methyl-parathion; 3, internal standard; 4, malathion; 5, parathion.

curves showed a linear relationship at a concentration range from 0.05 to 20 μg/ml (or μg/g) with correlation coefficients (R^2) from 0.9876 to 0.9980. For methyl parathion and malathion, there was linear relationship between 0.1 and 20 μg/ml (or μg/g) with correlation coefficients (R^2) from 0.9983 to 0.9999 and 0.9893 to 0.9999, respectively (Table 1).

The precision of the method was calculated as percent relative standard deviation at each biological specimen at three concentration levels (2, 4 and 8 μg/ml) applying four replicate analyses. The values obtained were lower than 10% in all cases. Table 1 gives the values obtained at the lowest concentration studied (2 μg/ml).

The absolute extraction recoveries were calculated to estimate the extraction efficiency. These were calculated by comparing the amount recovered by the HS-SPME method with the total amount initially added to the biological sample. The obtained values are summarised in Table 1. Recoveries varied significantly between the analytes and were also found to be sample dependent. The lowest recoveries were observed in liver and the highest recoveries in CSF. Liver is a very complex tissue, rich in proteins and other bio-macromolecules. In such a matrix, analyte mass transfer to the headspace may be hindered. In addition protein binding may occur, a phenomenon that could practically reduce the free analyte mass that is actually available for extraction and analysis. A

Table 1
Analytical figures of merit of the developed method correlation coefficients (R^2), limits of detection (LODs) percent relative standard deviation (R.S.D.%) ($n = 4$) and absolute recovery (%) obtained after application of SPME to the determination of four organophosphorus pesticides *in the studied* biological samples

	Dynamic range (μg/ml)[a]	Regression equation	R^2	LOD (ng/ml)[b]	R.S.D.% (2 μg/ml)[a]	Absolute recovery (%)
Whole blood						
Diazinon	0.02–20	$Y = 1.0385X + 0.3928$	0.9907	3	8	1.7
Methyl parathion	0.5–20	$Y = 0.0438X - 0.004$	0.9992	50	0.9	0.24
Malathion	0.1–20	$Y = 0.0552X - 8E{-}0.5$	0.9997	45	5	0.14
Parathion	0.02–20	$Y = 0.291X + 0.0913$	0.9914	18	3	0.67
Plasma						
Diazinon	0.02–20	$Y = 0.3391X + 0.1353$	0.9885	5	2	1.39
Methyl parathion	0.5–20	$Y = 0.0538X - 0.0116$	0.9988	40	9	0.23
Malathion	0.5–20	$Y = 0.0538X - 0.003$	0.9986	45	9	0.14
Parathion	0.02–20	$Y = 0.1416X + 0.00427$	0.9897	16	6	0.78
Kidney						
Diazinon	0.02–20	$Y = 0.2304X + 0.0699$	0.9961	4	4	1.34
Methyl parathion	0.5–20	$Y = 0.0498X - 5E{-}0.5$	0.9989	35	4	0.36
Malathion	0.5–20	$Y = 0.0201X - 0.0096$	0.9893	50	9	0.10
Parathion	0.02–20	$Y = 0.1007X + 0.0032$	0.9919	12	2	0.87
Liver						
Diazinon	0.02–20	$Y = 0.1342X - 0.0088$	0.9999	10	9	0.31
Methyl parathion	0.5–20	$Y = 0.0647X - 0.0022$	0.9983	55	3	0.16
Malathion	0.1–20	$Y = 0.024X - 0.0009$	0.9998	50	4	0.11
Parathion	0.02–20	$Y = 0.0863X + 0.0039$	0.9980	25	2	0.32
Cerebrospinal fluid						
Diazinon	0.02–20	$Y = 0.3513X + 0.1606$	0.9866	2	4	2.5
Methyl parathion	0.1–20	$Y = 0.0532X - 0.0018$	0.9999	35	6	0.35
Malathion	0.05–20	$Y = 0.0304X - 0.0007$	0.9999	40	8	0.28
Parathion	0.02–20	$Y = 0.1331X + 0.0505$	0.9876	8	6	1.25

[a] μg/g for liver and kidney tissue.
[b] ng/g for liver and kidney tissue.

solution to overcome such obstacles and enhance extraction efficiency could be to perform a systematic and thorough study of the matrix effect. However, this was not within the scope of the present study, which in fact is more focused on the applicability and the potential of the SPME in processing biological samples. Hence the conditions selected following optimization in "academic solutions" were adapted through the study for processing each biological specimen. However, further optimisation of the sampling procedure in each different sample matrix could improve extraction efficiency.

The differences in the extraction recovery resulted in differentiations on the limits of detection (LOD) values obtained for the same pesticide in different samples. LODs calculation was based on the lowest detectable peak that gave a signal to noise ratio of 3. The obtained values are also included in Table 1. It can be seen that the LODs are sample dependent. High LODs are observed in complex samples (liver), whereas low LODs are obtained in less complex samples (CSF). Between the pesticides, higher GC signals were observed for diazinon, thus as result low limits of detection were finally observed for this compound: 2–10 ng/ml (or ng/g). In contrast the signals obtained for methyl parathion and malathion were significantly lower, thus higher limits of detection were observed: 35–55 for methyl parathion and 40–50 ng/ml (or ng/g) malathion, respectively. For parathion the LODs ranged from 8 to 25 ng/ml (or ng/g).

4. Conclusions

The proposed methodology renders an efficient, cost effective and simple sample preparation process for the determination of OPPs in various biological specimens (liver, kidney, CSF, whole blood and blood plasma). The technique overcomes limitations and obstacles of conventional methods such as the use of expensive and toxic organic solvents and the application of tedious and cumbersome procedures. However, it is evident that systematic studies are necessary for each different biological specimen in order to suppress matrix effects and enhance extraction efficiency. In addition combination of HS-SPME with GC–MS would further enhance the method's potential, enabling positive analyte identification.

The HS-SPME mode was chosen for the processing of complex biological samples. The experimental procedure employed processing of a large number of real samples. The performance of the fiber was not practically affected by the various sample matrices and long fiber life times were observed (fibers actually failed due to coating corrosion or breakdown in the GC injection port). The overall method provided wide linear range, satisfactory detection sensitivity and repeatability thus enabling direct application in toxicological analysis of forensic samples. To our knowledge this is the first application of SPME on CSF analysis and the first report on the SPME–GC determination of OPPs in the samples kidney, CSF and liver. The present communication provides further evidence on the potential of HS-SPME in the processing of various biological tissue specimens for toxicological studies [19].

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