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Headspace solid-phase microextraction in combination with gas chromatography and tandem mass spectrometry for the determination of organochlorine and organophosphorus pesticides in whole human blood ☆

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Abstract

A method for the determination of several organochlorine and organophosphorus pesticides in human whole blood samples was developed. The combination of solid-phase microextraction in headspace mode with gas chromatography with tandem mass spectrometry allowed the determination of 11 selected pesticides at ppb levels, minimizing the sample treatment. Quantitation was carried out by means of calibration curves prepared in blood using labelled surrogate/internal standards. The method showed good linearity between 1 and 50 ng ml^{-1} (0.5–25 ng ml^{-1} for HCB) using second-order calibration curves. Precision was found to be better than 20% at the three concentration levels assayed in the range of ng ml^{-1}. The detection limits obtained were in the range 0.02–0.7 ng ml^{-1}, except for p,p′-DDT (3 ng ml^{-1}). The developed procedure was applied to blood and serum samples obtained from agricultural workers. HCB, β-HCH and p,p′-DDE were most frequently detected in the samples analyzed.

Keywords: Pesticides

1. Introduction

The widespread use of pesticides has posed problems for both our environment and health, exposure to these hazardous compounds being of great concern to the general public. Biological monitoring is a useful tool for assessing exposure to pesticides and involves the measurement of a biomarker of exposure (usually the pesticide or its metabolite) in human blood, urine or tissues, thus determining the internal dose of the toxicant [1]. There are several problems involved in the measurement of toxic compounds in biological samples, the most important being the low concentration levels involved and the complexity of sample matrices (urine, serum or whole blood). Therefore, biological monitoring requires reliable analytical methods for the accurate determination of pesticides and/or metabolites at the low levels found in these types of samples.

Measuring the internal dose of toxicants in blood

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has several advantages over measuring it in urine. Generally, the parent compound, instead of a metabolite, can be directly monitored in blood products such as whole blood, plasma, or serum; therefore, the development of a blood measurement technique usually does not require detailed information on the metabolism. The major disadvantages of blood measurements are the venipuncture required to obtain the sample and the low concentration levels. In addition, when samples can be obtained, the amount of blood available to perform the analysis is often limited. These facts stress the need for developing especially sensitive analytical procedures.

For the determination of pesticides in blood products, serum is usually preferred over whole blood due to the major complexity of the matrix in whole blood and because serum is a more homogeneous material [2]. However, the analysis of blood can provide interesting information on the total levels of some contaminants before their distribution in the different blood compartments. The analysis of whole blood samples usually requires tedious procedures including one or more clean-up steps in order to remove interferences: treatment with sulfuric acid after liquid–liquid [2,3] or solid-phase extraction (SPE) [4] has been used for the determination of organochlorine (OC) pesticides and PCBs in whole blood samples, sometimes combined with an additional SPE [2]. SPE clean-up has been also applied after hexane extraction for the determination of HCH isomers, aldrin and DDT metabolites in human blood [5]. Chlordane compounds and metabolite residues have been determined in whole blood using extraction with extrelute columns and SPE clean-up with florisil [6].

When the analytes are suitably volatile, an alternative for the analysis in these complex matrices is the extraction in headspace (HS) mode in order to avoid most interferences. The use of solid-phase microextraction (SPME), quite a recent extraction technique developed by Pawliszyn and co-workers [7–9], in the HS approach appears a feasible technique favored by a reduction in sample handling, even eliminating the need for clean-up steps. HS-SPME procedures have been proposed for the determination of different compounds such as cyanide [10], ethanol [11] and fenfluramine and amphetamines [12] in whole blood samples. With regards to pesticides, determination of OC [13] and organophosphorus (OP) pesticides [14–16], as well as dinitroaniline herbicides [17], has been reported in whole blood samples using HS-SPME.

Detection of extracted pesticides can be carried out by using different gas chromatography (GC) detectors, but it is preferable to use mass spectrometry (MS) detection in order to further assess peak identity. The use of MS detection clearly increases detection capabilities giving spectral identification of separated compounds. In spite of this, the use of typical EI-MS detection usually suffers from peak detection problems even when using a selected ion for quantitation, especially with very complex matrices such as biological fluids [18,19]. This situation can be overcome by using mass spectrometry in tandem detection with an adequate selection of precursor and daughter ions as referenced in the literature [20,21]. Thus, procedures based on a final determination by GC–MS–MS usually give good results both for identification and quantitation of pesticides in biological samples at low concentration levels.

In this paper, a study on the applicability of the HS-SPME method combined with GC–MS–MS for pesticide determination in whole human blood is reported. Development and optimization of SPME procedures were carried out separately for several OC and OP pesticides by using GC–ECD and GC–FPD, respectively. Later, a method was developed for both types of compounds using tandem mass spectrometry detection. Quantitation has been performed using calibration curves prepared by spiking blank blood samples, and using labelled surrogate standards. The developed multiresidue procedure has been applied to blood and serum samples from agricultural workers.

2. Experimental

2.1. Reagents

The 96.5–99.7% purity pesticide standards were purchased from Dr Erhenstorfer (Pomochem, Wesel, Germany) and used without further purification. Stock standard solution mixtures (~50 $\mu g\ ml^{-1}$) for OP and OC pesticides (except 25 $\mu g\ ml^{-1}$ for HCB) were prepared in acetone from stock individual standards and stored at −18 °C. Working solutions

were prepared by dilution with methanol and stored at 4 °C. Spiked water and blood samples were prepared by adding the appropriate volume of the methanol standard solution, maintaining a final constant concentration of 2.5% methanol, thoroughly shaking and allowing to equilibrate overnight at 4 °C.

4,4′-DDE D_8 (Dr Ehrenstorfer) and HCB-$^{13}C_6$ (Cambridge Isotope Labs, Andover, MA) were used as surrogate/internal standards. Stock solutions of labelled standard (~100 μg ml^{-1}) were prepared in acetone and stored at −18 °C. Working solutions were prepared by dilution with methanol.

Organic solvents (methanol and acetone) were for pesticide residue analysis (Scharlau, Barcelona, Spain). Sulfuric acid (Scharlau) and hydrochloric acid (J.T. Baker, Deventer, The Netherlands) were of analytical grade. Sodium chloride (Scharlau) of analytical grade was used after purification by heating at 300 °C overnight.

2.2. Samples

Blood samples used for optimization studies were obtained from non-exposed healthy subjects, while samples for analysis were collected from citrus growers. All of them were supplied by Gabinete de Seguridad e Higiene en el Trabajo de la Conselleria de Trabajo de la Generalitat Valenciana. The samples were collected into Vacutainer tubes (Sterile Interior brand) containing K_3 EDTA for avoiding coagulation and stored at −18 °C.

2.3. Instrumentation

The SPME device used for manual extraction, consisting of a holder assembly and several replaceable fibers, was purchased from Supelco (Spain). Three different fiber types were compared: non-polar polydimethylsiloxane (PDMS, 100 μm), more polar polyacrilate (PA, 85 μm) and Carbowax/divinylbenzene (CW/DVB, 65 μm). The fibers were conditioned as recommended by the manufacturer by heating them in the injection port of the chromatographic system during 0.5–2 h at 250−300 °C depending on the fiber type.

Analysis of OC pesticides was performed using a Hewlett-Packard 5890 Series II gas chromatograph (Avondale, USA) equipped with a splitless injector (2 mm I.D. glass liner) and electron capture detection (ECD) system. The GC system was fitted with a 25 m×0.2 mm I.D., 0.33 μm HP Ultra 2 (5% phenyl methyl siloxane) column. Injector temperature was 260 °C, while the oven temperature program used was: 120 °C (5.5 min), 30 °C min^{-1} to 150 °C, 3 °C min^{-1} to 270 °C with a final hold for 0.5 min. Helium was used as carrier gas at a flow-rate of 1 ml min^{-1}. ECD conditions: temperature 300 °C, nitrogen (make-up) 60 ml min^{-1}.

Analysis of OP pesticides was performed using an Ultra-Trace GC gas chromatograph (Fisons Instruments, Milan, Italy) based on the GC 8000 Series 2, equipped with an FPD-80 flame photometric detector and PC-based data system (Chrom Card) to control data acquisition and instrument conditions. The pressure of detector gases, hydrogen and air, was set at 140 and 60 kPa, respectively. Helium was used as carrier gas at a constant flow-rate of 1 ml min^{-1}. The analytical column was 30 m×0.32 mm I.D., with 0.50 μm 5% phenyl methyl siloxane (Hewlett-Packard). Detector and injector temperatures were 280 and 250 °C, respectively. The oven temperature was programmed as follows: 60 °C (5.5 min), 30 °C min^{-1} to 200 °C, 3 °C min^{-1} to 270 °C with a final hold time for 5 min.

GC–MS–MS was performed using an ion trap mass spectrometer (Finnigan GCQ, Austin, TX, USA) operating in electron impact (EI) ionization mode. The GC 8000 Top (CE Instruments, Milan, Italy) was equipped with a AS 800 autosampler. The oven temperature was programmed as follows: 90 °C (4 min), 30 °C min^{-1} to 180 °C, 4 °C min^{-1} to 270 °C, 30 °C min^{-1} to 300 °C with a final hold for 3.5 min. Splitless injections (3 mm I.D. glass liner) of 2 μl were performed using a 30 m×0.25 mm fused-silica DB-5MS capillary column with a film thickness of 0.25 μm (cross-linked 5% phenyl methyl siloxane). Helium was used as carrier gas at a flow-rate of 1 ml min^{-1}. The injector temperature was 240 °C. Experimental mass spectrometer conditions are summarised in Tables 1 and 2. Extracted ions selected from EI-MS–MS spectra for quantitation are indicated in Table 3.

2.4. Recommended analytical procedure

For the extraction of pesticides, 0.5 ml human EDTA blood, 25 μl of 400 ng ml^{-1} surrogate/internal standards mixture and 50 μl 4.5 *M* HCl

Table 1
Mass spectrometer conditions

Ionisation mode	EI (70 eV)
Multiplier voltage	1550 V
Multiplier gain	3.2×10^5
Source temperature	220 °C
Transfer line temperature	275 °C
Emission current	250 μA
AGC target	50
Trap offset	10

solution were placed into a 4-ml clear glass vial (36 mm×12 mm I.D.), and sealed with a silicon-lined septum screw cap. After shaking the vial for 1 min in a vortex, 0.5 ml ultrapure water were added. Before the extraction, the sealed vial was preheated at 90 °C with stirring in a silicone bath for 30 min. Then, the needle of the SPME device was passed through the septum and the PA fiber was pushed out from the needle and exposed to the HS of the vial for 30 min. After absorption, the fiber was allowed to dry for ~10 min. Then, it was thermally desorbed during 4 min into the glass liner of the GC injection port at 240 °C, maintaining initial oven temperature at 90 °C. Quantitation of samples was made using a calibration curve prepared by spiking blank blood samples and using isotopically labelled pesticides as surrogate/internal standards (HCB-$^{13}C_6$ for quantitation of HCB and p,p′-DDE-D_8 for the rest of pesticides). Stirring bars were heated at 300 °C

Table 2
Program for MS–MS detection of selected compounds

Compound	Parent ion	Time (min)	Window	Mass range	Voltage (V)
HCB	288	3.50	10	170–295	3.50
β and γ-HCH	219	10.60	5	140–225	1.05
Fonofos	246	11.00	1	120–250	0.70
Endosulfan-ether	241	11.60	8	165–245	1.56
Chlorpyrifos	316	13.67	5	250–320	1.20
α-Endosulfan	241	15.50	8	165–245	2.50
p,p′-DDE	250	16.50	10	145–260	2.65
p,p′-DDD	235	18.30	5	160–240	1.30
Ethion	231	18.70	1	120–240	0.80
p,p′-DDT	235	19.90	5	160–240	1.30

Table 3
List of ions (m/z) extracted in MS–MS chromatograms for quantitation (relative abundances %)

Compound	m/z
1. HCB	212–216 (214, 100%), 249–253 (249, 55%), 284 (30%), 286 (25%)
2. β-HCH	181 (100%), 183 (85%)
3. γ-HCH	181 (84%), 183 (100%)
4. Fonofos	137 (100%), 174 (12%), 202 (23%)
5. Endosulfan-ether	204–208 (206, 100%), 237–241 (239, 54%)
6. Chlorpyrifos	258 (100%), 260 (92%), 286 (85%), 288 (48%)
7. α-Endosulfan	170 (73%), 172 (45%), 204–208 (204, 100%)
8. p,p′-DDE	175 (16%), 176 (98%), 177 (16%), 246 (100%), 248 (46%)
9. p,p′-DDD	165 (100%), 199 (36%)
10. Ethion	175 (100%), 203 (68%)
11. p,p′-DDT	165 (100%), 199 (6%)
Labelled surrogate/internal standard	
$^{13}C_6$ HCB	218–222 (220, 100%), 253–257 (255, 72%), 288 (70%)
D_8 p,p′-DDE	183 (80%), 184 (100%), 185 (15%), 254 (72%)

during 1 h between experiments in order to avoid carry-over.

3. Results and discussion

3.1. HS-SPME optimization

Before the application of HS-SPME to blood samples, several experiments with spiked water samples were carried out in order to select the optimum conditions for the extraction process. Determination of OC and OP pesticides in this first step was performed separately by GC–ECD and GC–FPD, respectively.

For the selected OC pesticides (HCB, lindane, endosulfan ether, α-endosulfan and p,p′-DDE) an optimization study on the desorption step was initially carried out. The best results were established as follows: desorption time 2 min at 260 °C with an initial oven temperature of 120 °C. PDMS, PA and CB/DVB fibers were compared for the extraction of selected OC pesticides from spiked water samples (10 ng ml^{-1} level) at two extraction temperatures: 45 and 90 °C (extraction time 30 min). Although some compounds such as HCB showed lower affinity for the fiber coating at higher temperature, in general extraction efficiency was better at 90 °C. With regard to the type of coating, the higher efficiencies corresponded to PA fiber.

Extraction times were studied using spiked water samples and the equilibrium was estimated to take up to several hours (ranging between 0.5 and 11 h). In this situation a fixed extraction time of 30 min was selected, being similar to the pre-heating time, thus simplifying the procedure and giving a total time of sample treatment comparable to the chromatographic run time. In this way a maximum sample throughput with manual extraction was obtained.

Once the conditions for fiber type, heating temperature and extraction time using spiked water samples were established, the HS-SPME procedure was applied to blood samples. The sample volume available when dealing with this type of sample is often limited, usually being less than 1 ml. Moreover, it is advisable to dilute blood samples with distilled water before extraction in order to reduce their complexity and avoid coagulation processes when the sample is heated. Therefore, in further experiments, a volume of 0.5 ml of whole blood diluted with 0.5 ml of ultrapure water was selected for developing the HS-SPME procedure for the determination of OC pesticides.

According to the available literature, analytical procedures for determination of pesticides in serum and blood samples usually include acidification of the sample before the extraction step, even when using SPME procedures [12–14]. Therefore, the effect of adding acid to the blood sample together with the effect of the ionic strength on the extraction efficiency was studied. So, 50 μl of sulfuric or hydrochloric acids at two concentration levels (9 and 4.5 *M*) and uni and divalent (sodium chloride and ammonium sulfate) salts (between 0 and 15%, w/v) were added to diluted blood samples. As an example, Fig. 1 shows the ECD response of some OC analytes after SPME of blood samples spiked at the 10 ng ml^{-1} level in different conditions of salt and acid concentration. As can be seen, the addition of H_2SO_4 9 *M* to the sample generally improved the response for the pesticides studied, allowing the determination of lindane which was not recovered without acid. Only HCB showed a notable decrease in its response with the addition of acid or salt. On the other hand, addition of sodium chloride and ammonium sulfate decreased extraction efficiency for all pesticides, as has also been reported by several authors [13,16]. This lower extraction efficiency following salt addition has been explained by the stabilization of the erythrocyte membranes by the salts, which causes trapping of the pesticides into membranous vesicles and thus their low recovery [13]. In spite of this, the addition of low salt concentrations (<10%) has been recommended in the literature in order to homogenize the salt content of samples despite lower pesticide extraction [22]. In our work, the use of hydrochloric acid instead of sulfuric acid or lowering acid concentration did not improve the results (Fig. 1) and we selected the addition of only 50 μl of 9 *M* H_2SO_4, which allowed us to achieve a better response for most compounds.

In relation to OP pesticides, preliminary experiments with spiked water samples using different fiber coatings showed that the highest extraction efficiencies were also obtained with PA fiber. Desorption conditions were established according to our previ-

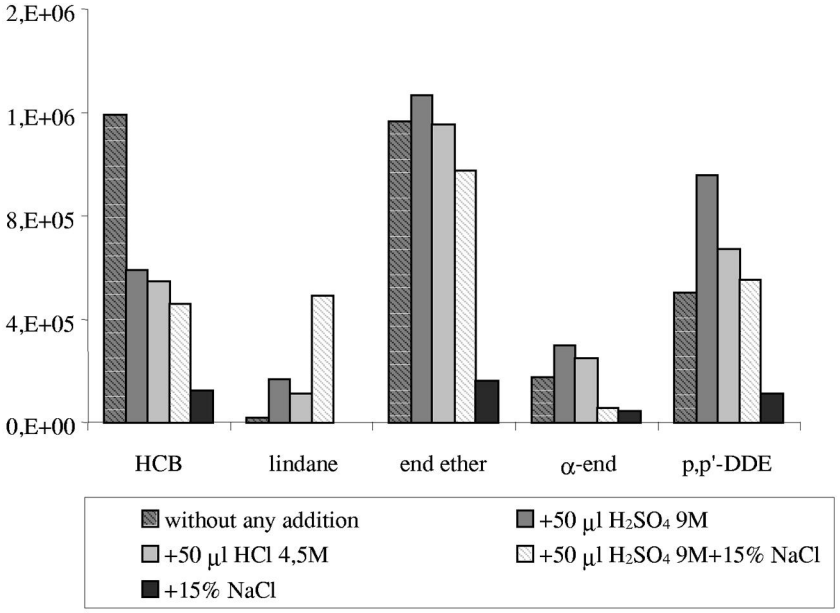

Fig. 1. Influence of the addition of acid and salt to blood samples fortified at 10 ng ml^{-1} on HS-SPME efficiency.

ous data for the determination of OP pesticides in water samples by direct immersion SPME [23] (4-min desorption time at 250 °C in the injector and initial oven temperature 60 °C). Initially, the experiments were carried out with eight OP pesticides, but soon it was evident that some of them (dichlorvos, phorate, methidation, azinphos methyl and metamidofos) were not suitable for HS extraction, probably due to their low volatility. Therefore, only fonofos, chlorpyrifos and ethion were eventually selected for carrying out the study.

The effects of the ionic strength and the acid addition on the OP pesticides extraction efficiencies were studied using blood samples spiked at the 40 ng ml^{-1} level. Addition of salt also led to lower extraction efficiencies. When studying the addition of different acids and concentrations, the best results were obtained when 50 μl of HCl 4.5 *M* were added to the sample, probably due to the low stability of OP pesticides in stronger acid conditions (as an example, fonofos and ethion are hydrolyzed in acid conditions [24]).

It is interesting to note that during some of the experiments, we found quantitation problems for most of the compounds at the lower spiking levels which were attributed to contamination due to the absorption and reloading of some of the compounds from the Teflon stirring bars. Some cleaning procedures were assayed to avoid memory effects between consecutive experiments, such as cleaning the bars with a saturated solution of potassium dichromate in sulfuric acid for 15 min, in *n*-hexane for 1 h or baking them at 200 °C for 1.5 h. Finally, pesticides were satisfactorily removed from stirring bars by heating at 300 °C for at least 1 h. After this efficient clean-up, all chemical blanks were free of the target analytes.

3.2. GC–MS–MS optimization

After optimization of the HS-SPME for selected OC and OP pesticides, the next step was to develop a multiresidual procedure for the simultaneous determination of both OC and OP pesticides in whole blood samples, carrying out subsequent analysis by GC–MS–MS. Besides the five OC and the three OP pesticides selected, β-HCH, p,p′-DDD and p,p′-DDT were also added to the standard mixtures in order to check their behavior. The use of MS–MS allowed us to increase the number of compounds to be studied

due to its improved sensitivity and, especially, its selectivity. For acidification of the sample before the extraction step, HCl was chosen for the multiresidue determination of OC and OP pesticides, as a compromise for both families of compounds. Therefore, the HS-SPME procedure was applied as indicated in Section 2.4.

The MS–MS method applied uses EI ionization mode with automatic gain control (AGC), which is characteristic of ion trap MS analyzer. MS–MS detection was performed by isolation of the corresponding selected parent ion for each compound inside the trap followed by application of an adequate excitation voltage for its subsequent fragmentation. Parent ions were selected from the EI spectra taking into account aspects such as high m/z values and also the peak abundance as well as the chromatographic signal obtained after its isolation in the ion trap. Daughter ions obtained were scanned over a characteristic mass range leading to the MS–MS spectrum. The object was to generate MS–MS spectra where the parent ions were present in at least 10–20% of base peak.

In this study, the MS–MS method was divided into ten acquisition segments (one for each pesticide, except for the isomers β-HCH and lindane). Three values of the trapping parameter q (0.45, 0.35 and 0.25) were investigated. The higher response for most compounds was obtained with $q=0.45$, so this value was selected for our method. Then, isolation and excitation conditions were optimized and selected in order to obtain characteristic MS–MS spectra with fragmentation patterns rich enough to allow accurate identification of analytes detected.

Table 2 shows the conditions corresponding to the optimized MS–MS method. The highest isolation windows used corresponded to HCB and p,p′-DDE as the labelled HCB-$^{13}C_6$ and p,p′-DDE D_8 were also included in these segments. In addition, the selected precursor ions in these isolation windows did not correspond to any base peak in their EI spectrum (284 for HCB and 246 for p,p′-DDE), but the precursor ion and window were selected to keep isolation mass range as short as possible and including the pesticide as well as the isotopically labelled compound. In general, OP compounds required lower values of fragmentation voltages (0.70 V for fonofos, 1.20 V for chlorpyrifos and 0.80 V for ethion) than OC pesticides, the highest voltages corresponding to HCB, α-endosulfan and p,p′-DDE (3.5, 2.5 and 2.65, respectively).

Extracted ions selected from EI-MS–MS spectra for quantitation are indicated in Table 3.

3.3. Analytical characteristics and validation

Once the experimental conditions were optimized, validation of the overall analytical procedure, including HS-SPME (PA) and the subsequent determination of selected OC and OP pesticides by GC–MS–MS was carried out as regards as linearity range, precision, accuracy and limits of detection.

Quantitation was performed using calibration curves prepared in matrix, by spiking blank blood samples with selected pesticides, and using labelled surrogate/internal standards. The blank blood sample consisted of a pool obtained by mixing ~20 individual EDTA blood samples. This pool was previously analyzed ($n=5$) and was found to contain HCB and p,p′-DDE (mean concentrations of 2.7 and 3.7 ng ml^{-1}; relative standard deviations of 20 and 12%, respectively), compounds that are usually detected in the human population. Therefore, it was necessary to correct the quantitative results in the samples by subtracting blank values (peak areas) for these two pesticides. Fig. 2 shows a typical chromatogram of this blank blood sample. Chemical blanks were free of the target analytes and, consequently, the presence of HCB and p,p′-DDE in the blank samples was

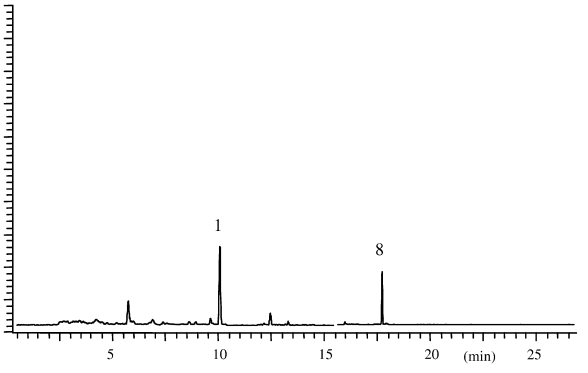

Fig. 2. Extracted ion GC–MS–MS chromatogram obtained after HS-SPME of a blank blood sample. 1, HCB; 8, p,p′-DDE (see Table 3 for m/z used).

assumed to be due to their ubiquitous presence in the environment and lipids of human beings.

Initially, some quantitation problems were observed, and were attributed to the fact that $^{13}C_6$ HCB was used as surrogate/internal standard for quantitation of the early eluting peaks (HCB, β-HCH, lindane, fonofos and endosulfan ether) and D_8 p,p′-DDE for the late eluting peaks (chlorpyrifos, α-endosulfan, p,p′-DDE, p,p′-DDD, ethion and p,p′-DDT). Coefficients of variation obtained in this way were too high (>20% in most cases at all fortification levels assayed) as matrix effect correction was inadequate. After several experiments, we decided that the best option was to use $^{13}C_6$ HCB only for HCB and D_8 p,p′-DDE for the rest of pesticides.

Linearity of the method was tested on blood samples spiked at the range of 1–50 ng ml^{-1} using five concentration levels (1, 5, 10, 25 and 50 ng ml^{-1}, except for HCB, which was 0.5, 2.5, 5, 12.5 and 25 ng ml^{-1}) and analyzing each level in triplicate. In the particular cases of HCB and p,p′-DDE, the responses used in the calibration curves were obtained by subtracting those found in the blank sample from their peak areas (relative to the internal labelled standard). A best fit of the experimental points was obtained when using second-order calibration curves with r^2 higher than 0.99 for all compounds in the whole range tested.

The precision and accuracy of the procedure were obtained by analysis of six spiked blood replicate samples at three concentration levels (1, 5 and 25 ng ml^{-1}) (Table 4). Most of calculated concentrations fell within the range of 90–120% of the spiking concentrations, with a few exceptions. In the case of fonofos, values obtained were above 120% at the three spiking levels assayed; chlorpyrifos also showed high values, especially at 5 ng ml^{-1} (143%). This problem could have possibly been corrected using better labelled internal standards (ideally fonofos and chlorpyrifos labelled standard). Ethion showed the worst accuracy, the calculated concentrations being lower than the spiking values (74%) at 5 ng ml^{-1} spiking level. Precision, expressed as coefficients of variation, was in general better than 20% and near this value for most of the compounds at the lowest concentration level assayed (1 ng ml^{-1}). The worst values were obtained for lindane and p,p′-DDT, in the latter compound probably due to the fact that the fortification level was close to the limits of detection. These coefficients of variation are quite similar to those reported for determining dinitroaniline herbicides (0.1–10 ng ml^{-1}) in blood and urine samples by SPME (~14%) [17], and are in the usual range of precision for SPME procedures when applied to the determination of pesticides in complex matrix samples [22,25]. Compared to meth-

Table 4
Accuracy and precision (n=6) for the overall HS-SPME and GC–MS–MS procedure for the determination of pesticides in spiked blood samples (limits of detection: ng ml^{-1})

	Spiking level[a] 25 ng ml^{-1}		Spiking level[b] 5 ng ml^{-1}		Spiking level[c] 1 ng ml^{-1}		LOD (ng ml^{-1})
	Mean concentration (ng ml^{-1})	C.V. (%)	Mean concentration (ng ml^{-1})	C.V. (%)	Mean concentration (ng ml^{-1})	C.V. (%)	
HCB	13.4	11	2.4	10	0.5	21	0.1
β-HCH	25.0	17	5.4	13	1.0	20	0.1
Lindane	23.5	20	6.1	22	1.0	35	0.2
Fonofos	30.8	10	6.5	14	1.3	16	0.4
Endosulfan ether	27.0	8	5.3	5	0.9	8	0.1
Chlorpyrifos	28.0	18	7.2	17	1.3	14	0.2
α-Endosulfan	28.8	8	5.2	13	1.2	9	0.5
p,p′-DDE	29.8	7	5.7	20	1.2	18	0.02
p,p′-DDD	28.8	11	5.4	19	1.0	22	0.4
Ethion	25.5	8	3.7	15	1.1	22	0.7
p,p′-DDT	31.3	20	5.2	41	–	–	3

[a] 12.5 ng ml^{-1} for HCB.
[b] 2.5 ng ml^{-1} for HCB.
[c] 0.5 ng ml^{-1} for HCB.

ods other than SPME used for the determination of pesticides in biological fluids at the ppb level, the precision obtained in the present paper is of the same order [26,27].

Between-day precision ($n=9$) was also studied by analyzing three replicates of blood samples spiked at two concentration levels (1 and 25 ng ml^{-1}) on 3 different days covering a total period of 5 days. Coefficients of variation were below 30% at both levels.

The limits of detection (LOD) were estimated from chromatograms (using extracted ion chromatogram) corresponding to blood samples spiked at the lowest level and calculated as the peaks having a signal-to-noise ratio of 3 (Fig. 3). As can be seen in Table 4, experimental LOD were in the range of 0.02–0.7 ng ml^{-1}, except for p,p′-DDT (3 ng ml^{-1}). Due to the presence of HCB and p,p′-DDE in all the samples analysed, including the blank sample, LOD for these compounds were estimated from the chromatograms corresponding to the measured sample with the lowest concentration (sample 15: HCB 0.7 ng ml^{-1} and p,p′-DDE 0.4 ng ml^{-1}). LOD for HCB and p,p′-DDE were found to be in the range of 0.1 and 0.02 ng ml^{-1}, respectively.

These LOD were similar or better than those found in the literature. Thus, Röhrig et al. [13] also used HS-SPME for the determination of OC pesticides in human blood reaching LOD of 0.15 ng ml^{-1} for lindane, 0.25 ng ml^{-1} for p,p′-DDD and 1.59 ng ml^{-1} for p,p′-DDT; for β-HCH, HCB and p,p′-DDE their LOD were slightly higher than those obtained in this paper (0.9, 0.8 and 0.08 ng ml^{-1}, respectively, compared to 0.1, 0.1 and 0.02 ng ml^{-1}). In the same way, using the same chromatographic detection technique (GC–MS–MS) and solvent extraction (with two clean-up steps) for the determination of endosulfan in serum, LOD were very similar (endosulfan-ether 0.05 ng ml^{-1} and α-endosulfan 0.3 ng ml^{-1}) [28]. Other authors found much higher LOD (25–30 ng ml^{-1}) for the determination of OC pesticides in human plasma by means of LLE with a Florisil clean-up step [29].

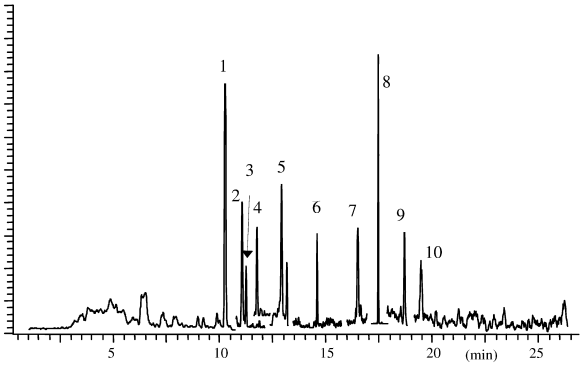

Fig. 3. Extracted ion GC–MS–MS chromatogram obtained after HS-SPME of a blood sample spiked with OC and OP pesticides at 1 ng ml^{-1}. 1, HCB; 2, β-HCH; 3, lindane; 4, endosulfan ether; 5, fonofos; 6, chlorpyrifos; 7, α-endosulfan; 8, p,p′-DDE; 9, p,p′-DDD; 10, ethion (see Table 3 for m/z used).

3.4. Application to real blood and serum samples

The procedure developed in this paper was applied to two groups of samples: 17 samples collected from growers from the province of Valencia (Spain) and five samples from pesticide applicators from the province of Castellon (Spain) engaged in citrus fruit production. Due to the limited volume of sample available, only one replicate of each sample could be analyzed. Tables 5 and 6 show the results obtained.

HCB (ranging between 0.7 and 4.3 ng ml^{-1}) and p,p′-DDE (0.4–74 ng ml^{-1}) were detected in all the blood samples, showing parallel behavior because higher concentrations of HCB usually corresponded to samples also containing high levels of p,p′-DDE. Samples 11, 12 and 14 are significant, containing the highest concentrations of HCB (~4 ng ml^{-1}) and p,p′-DDE (48, 74 and 66 ng ml^{-1}). Of the 22 samples analyzed, number 12 was by far the most contaminated, with the highest concentrations of p,p′-DDE, HCB and β-HCH. Additionally, it was the only blood sample where we detected p,p′-DDT and p,p′-DDD. β-HCH was detected in seven samples at concentrations always ~1 ng ml^{-1}.

As depicted in Table 5, two samples contained p,p′-DDE at concentrations above linearity of the method (50 ng ml^{-1}). In these cases, we estimated their concentrations at ~66 and 74 ng ml^{-1}, as the calibration curve was actually tested up to 100 ng ml^{-1}, although linearity was only established up to 50 ng ml^{-1}.

These findings are in agreement with different monitoring surveys performed in human population, where OC pesticides such as HCB, p,p′-DDT and

Table 5
Concentrations (ng ml^{-1}) of pesticides detected in human blood from a group of 17 growers from Valencia

	Sample number																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
HCB	2.2	1.2	2.1	2.4	1.9	2.8	1.7	1.1	1.1	1.0	3.8	4.3	1.7	4.1	0.7	2.0	0.8
β-HCH	1.4	–	–	–	–	1.5	–	–	–	–	1.7	1.8	1.1	1.2	–	–	1.4
Chlorpyrifos	–	–	–	–	d[a]	–	–	–	–	–	–	–	–	–	–	–	d
p,p′-DDE	12	3.0	6.2	9.3	2.2	2.8	5.3	27	14	21	48	74[b]	22	66[b]	0.4	8.1	8.8
p,p′-DDD	–	–	–	–	–	–	–	–	–	–	–	0.8	–	–	–	–	–
p,p′-DDT	–	–	–	–	–	–	–	–	–	–	–	5.0	–	–	–	–	–

[a] d, detected.
[b] Estimated value.

metabolites and HCHs are frequently detected in blood (serum) or adipose tissues. In a previous work [30], we also detected HCB, p,p′-DDT, and p,p′-DDE in human serum samples.

The OP insecticide chlorpyrifos (widely used nowadays on citrus crops of the Mediterranean region) was detected in four samples, in all cases at concentration levels below or around the limit of detection of the procedure (0.2 ng ml^{-1}). The presence of chlorpyrifos in blood should be due to recent exposure of growers to this compound because it is rapidly metabolized in humans, being excreted mainly as the metabolite 3,5,7-trichloro-6-pyridinol in urine.

As an example, Fig. 4 shows the extracted ion GC–MS–MS chromatogram corresponding to blood sample 12.

In a previous paper [30], we developed a procedure for the determination of OC and OP pesticides in human serum using HS-SPME followed by GC–MS. In the present work, we have used optimized GC–MS–MS detection, but maintaining the same extraction conditions originally established [28] (1 ml serum+50 μl 9 *M* H_2SO_4+2 ml distilled water; HS extraction for 30 min at 90 °C of stirred samples with PDMS fiber). This new procedure was checked for linearity range, which was tested over a range of 1–25 ng ml^{-1} in serum sample (0.2–12.5 ng ml^{-1} for HCB). The best fitting of the experimental points corresponded to second-order calibration curves with r^2 higher than 0.99 for all compounds in the whole range tested. Limits of detection were also calculated from the GC–MS–MS (extracted ion) chromatograms of serum samples spiked at the lowest level (1 ng ml^{-1}). Experimental LOD were in the range of 0.03–0.3 ng ml^{-1} (1 ng ml^{-1} for β-HCH and 1.6 ng ml^{-1} for α-endosulfan). In this way, LOD obtained with MS–MS detection were more satisfactory than those of MS for most compounds. For example, the LOD for lindane and p,p′-DDT using MS detection were found to be 1 and 5 ng ml^{-1}, respectively, while for MS–MS detection the LOD was 0.2 ng ml^{-1} for both compounds.

This multiresidual procedure was applied to serum samples from the five pesticide applicators whose

Table 6
Concentrations (ng ml^{-1}) of pesticides detected in human blood and serum from a group of five pesticide applicators (citrus crops) from Castellon

	Sample number									
	18		19		20		21		22	
	Blood	Serum	Blood	Serum	Blood	Serum	Blood	Serum	Blood	Serum
HCB	2.5	3.4	2.8	4.8	2.2	3.5	1.8	2.5	1.8	2.6
Chlorpyrifos	d[a]	0.7	0.4	0.6	–	–	–	–	–	–
p,p′-DDE	8.7	13	13	27	3.1	4.8	8.1	16	5.6	7.0

[a] d, detected.

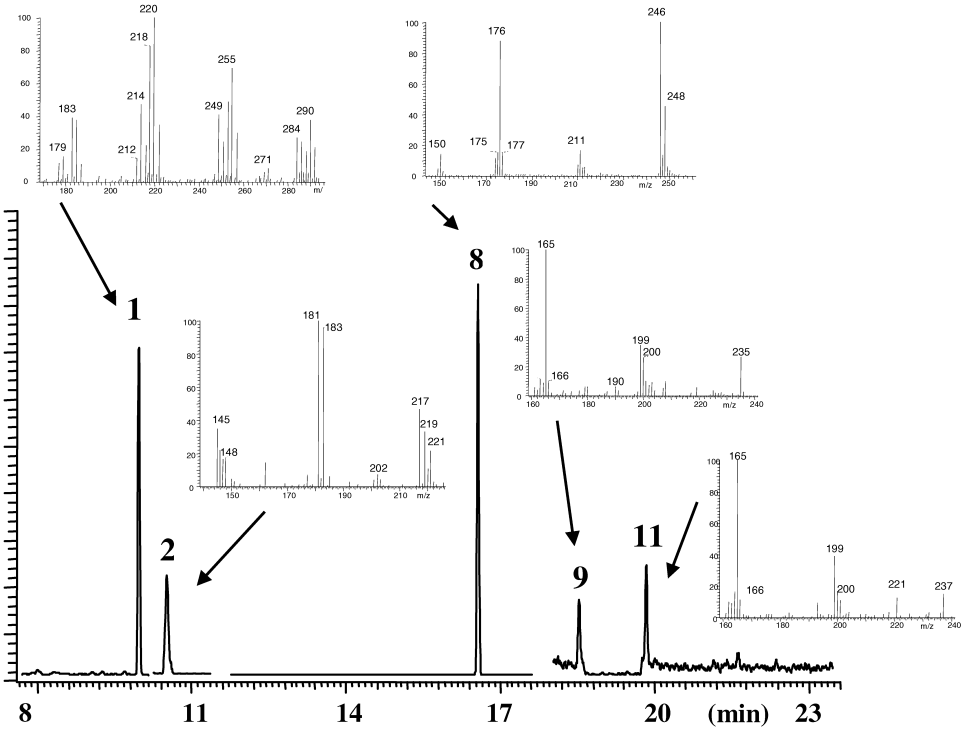

Fig. 4. Extracted ion GC–MS–MS chromatogram of blood sample 12. 1, HCB; 2, β-HCH; 8, p,p′-DDE; 9, p,p′-DDD; 11, p,p′-DDT (see Table 3 for m/z used).

whole blood had been analysed before. Results obtained are in agreement with those of whole blood (Table 6), although concentration levels in serum were always higher than in blood by a factor ranging from 1.3 to 2.1. This increase in the values could be due to the volume concentration that occurs when separating serum from whole blood or to the different distribution of organic compounds in blood compartments.

Fig. 5 shows the extracted ion GC–MS–MS chromatogram corresponding to serum sample 18.

4. Conclusions

The SPME procedure developed in this paper allows an important reduction in sample treatment compared with other conventional techniques such as liquid–liquid and solid-phase extraction. Although SPME is usually applied in the direct immersion mode, we carried out extraction in the HS mode due to the complexity of blood sample matrix. Thus, the use of calibration curves prepared with spiked blank blood provides accurate results for quantitation, isotopically labelled surrogate/internal standards also being required to correct the matrix effect. Moreover, the application of GC–MS–MS provides a satisfactory sensitivity and selectivity avoiding most of the interferences from this type of complex biological matrix.

The overall SPME–GC–MS–MS procedure allowed us to determine several OC and OP pesticides simultaneously in whole blood leading to low detection limits for most of the pesticides studied (0.02–0.7 ng ml^{-1}). The method can be suitable for detecting pesticides of different physico-chemical characteristics in exposed and non-exposed population and can be widened to the analysis of serum samples.

The developed procedure has been applied to a number of blood and serum samples where several OC pesticides were detected.

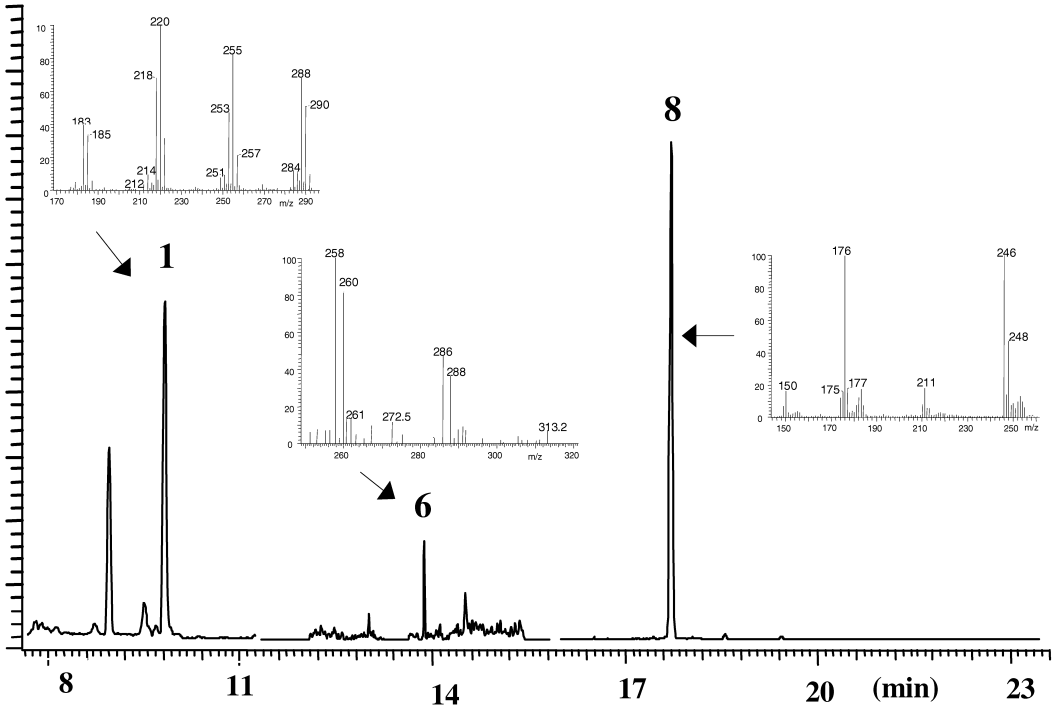

Fig. 5. Extracted ion GC–MS–MS chromatogram of serum sample 18. 1, HCB; 6, chlorpyrifos; 8, p,p′-DDE (see Table 3 for m/z used).

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