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High throughput method for the determination of organochlorine pesticides and polychlorinated biphenyls in human serum

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Abstract

An improved method for the determination of selected organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in human serum was developed. The method requires low volume of serum (500 μl) and 48–96 samples per day can be prepared by one analyst without special automatic equipment. Initial extraction was performed using 96-well solid-phase extraction disk plates and was followed by a clean-up with silica gel/sulfuric acid. Different denaturation, elution and clean-up conditions were tested. Quantification was carried out by gas chromatography equipped with electron capture detector (GC-ECD) or mass spectrometer (GC–MS). Recoveries of PCB congeners 28, 52, 101, 118, 138, 153 and 180 and OCPs HCB, β-HCH, *p,p′*-DDE and *p,p′*-DDT at two spiking levels ($n = 8$) varied from 57 to 120%, and intra-day relative standard deviation from 1 to 11%, both depending on spiking level and compound. Inter-day relative standard deviation was <15% in all cases. Limit of quantification (LOQ) for these PCBs ranged from 0.08 to 0.13 ng/ml and for these OCPs from 0.16 to 0.40 ng/ml. The optimized method was applied to the analysis of 1000 serum samples from different places of Spain.

Keywords: Organochlorine pesticides; Polychlorinated biphenyls (PCBs); 96-Well solid-phase disk extraction plate; Gas chromatography; Human serum

1. Introduction

Organochlorine pesticides (OCPs) have been extensively used in agriculture, mainly as insecticides, while polychlorinated biphenyls (PCBs) are been widely used in transformers, capacitors and paper and paints industry. Both groups of compounds are highly lipophilic, chemically very stable and resistant to environmental degradation, and consequently they are considered to be persistent organic pollutants (POPs) in environment. Nine of the OC pesticides, as well as PCBs, were the subjects of the Stockholm convention on POPs. The proposed treaty called for urgent global actions to reduce and eliminate releases of these compounds [1]. However, although most of them are banned for several decades and no longer used, they are globally spread in environment and may be routinely found in seawater, air, fish, wildlife, food and even humans. They or their metabolites remain as residues and due to their lipophilic characteristics they bioaccumulate in fatty tissues getting up to the human organism trough the diet, especially foods of animal origin. In fact, even though occupational or direct environmental exposure may affect selected groups of population, chronic low dose exposure through diet is the main concern for general population as it poses a risk of causing adverse effects to human health. PCBs have been shown to cause cancer in animals and other non-cancer effects, since they could affect the immune, reproductive, nervous and endocrine systems. Studies in humans provide supportive evidence for these potential carcinogenic and non-carcinogenic effects [2–5]. PCBs are been rated by International Agency of Research on Cancer (IARC) as "probably carcinogenic to humans" (2A group), while most OCPs were classified as "possibly carcinogenic to humans" (2B group) [6,7]. So, they raise especial interest in public health and epidemiology.

As it is very difficult to estimate the exposure and intake of such compounds from standards questionnaires, biomarkers must be considered a reasonable alternative. Measurements of

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the OCPs and PCBs or their metabolites in body tissues and fluids (biological monitoring) have been done as useful approach for assessing the exposure risk in the epidemiological studies. Human serum is one of the biological materials that can be conveniently obtained and used for these types of studies [8–11]. Conventional methods for determination of PCBs and OCPs in serum involve liquid–liquid extraction (LLE) using organic solvents [12–16] or solid-phase extraction (SPE) by columns or cartridges [17–20], followed by clean-up steps to remove interferences. The cleaned extract is later analyzed using capillary GC with electron capture detector (ECD) [15,16,19,21] or capillary GC with MS detection [15,20–23]. Generally, methods based on LLE are complex, time-consuming and use excessive amounts of solvents and reagents, becoming inadequate for the number of samples required for epidemiological studies [17,24]. SPE methods with columns or cartridges are considered to be superior due to their advantages of simplicity, reduced solvent usage and better throughput [17,18,24]. However, they require no less than 5–10 ml of solvent to complete the extraction steps, usually more than 2 ml of serum, and one analyst rarely can prepare above 15 samples per day.

The present work was focused on developing an improved method for routine determination of selected OCPs and PCBs in human serum using 96-well solid-phase extraction disk plates. This procedure combines the advantages of SPE disks (reduced elution volumes and good performance) with the high throughput of 96-well plates, thus giving rise to a method that fulfils the requirements for bio-monitoring human exposure to selected OCPs and PCBs in epidemiological studies. Different denaturation, elution and clean-up conditions were tested in the experimental stage to optimize the method. The optimized procedure was successfully applied to the analysis of 1000 serum samples from different places of Spain.

2. Experimental

2.1. Reagents and materials

Standard solutions PCB "Key" (28, 52, 101, 138, 153 and 180) and PCB "Dioxin Like" (77, 81, 105, 114, 118, 123, 126, 156, 157, 169 and 189), each at 10 ng/μl in isooctane, and PCB 46 and 143 solutions (internal standards), each at 100 ng/μl in hexane, were purchased from LGC-Promochem (Middlesex, UK). Organochlorine pesticides (HCB, α, β, γ and δ-HCH, heptachlor, heptachlor epoxide, *o*,*p*′-DDT and metabolites, *p*,*p*′-DDT and metabolites, α, and β-endosulfan, aldrin, endrin, dieldrin and methoxychlor) neat standards were supplied by Dr. Ehrenstorfer (Augsburg, Germany).

Acetonitrile and methanol HPLC gradient grade were purchased from Scharlab (Barcelona, Spain). *n*-Hexane, dichloromethane, cyclohexane and toluene GC grade together with silica gel 60 column chromatography grade and glass wool were from Merck (Darmstadt, Germany). Concentrated H_2SO_4 analytical grade and anhydrous Na_2SO_4 pesticide grade were obtained from Panreac (Barcelona, Spain). Empore™ 96-well SPE disk plates (C18, 1 ml) and vacuum manifold from 3 M (St. Paul, MN, USA), and 96-glass microdilution tube recollection racks from Marsh Bio Products (Rochester, NY, USA). TurboVap LV evaporator was supplied by Zymark Corp. (Hopkinton, MA, USA).

PCB stock mixture solutions were prepared in cyclohexane and OCP stock mixture solutions in toluene. These solutions were stored at −20 °C. From these mixtures, calibration solutions were made in cyclohexane and spiking solutions in acetonitrile. These solutions were stored at 4 °C.

Silica-sulfuric acid mixture for clean-up was prepared mixing 5 ml of sulfuric acid, drop by drop and stirring, with 10 g of silica gel. Once the acid was added, the mixture was stirred until good homogeneity was got.

Glass wool, Na_2SO_4 and silica gel were used after heating overnight at 300 °C. All glassware was washed with detergent, rinsed with water, heated overnight at 300 °C and rinsed with dichloromethane or hexane before use.

2.2. Serum samples

Serum used for development and validation of the method was obtained from EPIC-Spain and volunteers of Public Health Laboratories of Guipuzcoa and Biscay. Blood was extracted by venipuncture and collected on Vacutainer blood tubes. Serum was separated by centrifugation. A pooled serum was made from 35 individual serum samples and was named as Laboratory-Serum (LS). All serum samples were kept frozen at −20 °C until analysis. To minimize thawing and refreezing, LS pooled serum was aliquoted in 10 ml glass tubes equipped with Teflon-lined screw caps.

2.3. Instrumentation

Quantification was performed on a 5890 series II gas chromatograph equipped with split-splitless injector, ECD detector, and a 7673 autosampler (Agilent Technologies, Wilmington, DW, USA). One microliter of sample was autoinjected in the splitless mode, with the split-splitless purge valve opened at 1 min after injection. The injection port temperature was 250 °C with helium as carrier gas at 135 kPa. Detector temperature was 300 °C with N_2 as make-up gas. Chromatographic separation was accomplished on a dual column system with a 30 m × 0.25 mm i.d. × 0.25 μm film thickness DB-XLB column (J&W Scientific, Folsom, CA, USA) for PCBs and a 50 m × 0.25 mm i.d. × 0.25 μm film thickness DB-5MS column (J&W Scientific) for OCPs. The oven program was started at 60 °C held for 1 min, programmed at 20 °C/min to 180 °C, then programmed at 1.5 °C/min to 240 °C, and finally programmed at 30 °C/min to 300 °C and held 8 min.

Quantitative and qualitative confirmation was carried out on a 6890 gas chromatograph equipped with EPC split-splitless injector, 5973 quadrupole MS detector, and a 7683 autosampler (Agilent Technologies). Two microliter of sample was autoinjected in the splitless mode, with the split-splitless purge valve opened at 1.2 min after injection. The injection port temperature was 270 °C with helium as carrier gas at 140 kPa. Chromatographic separation was accomplished on a 30 m × 0.25 mm i.d. × 0.25 μm film thickness DB-5MS column (J&W Scien-

Table 1
Masses used for GC–MS confirmation after GC-ECD determination

Analyte	Target ion (*m/z*)	Qualifier ion (*m/z*)
Trichlorobiphenyl	258	260
Tetrachlorobiphenyl	290	294
Pentachlorobiphenyl	326	324
Hexachlorobiphenyl	360	290
Heptachlorobiphenyl	394	396
HCB	284	282
β-HCH	183	217
Heptachlor epoxide	353	355
p,p′-DDE	246	318
p,p′-DDT	235	237

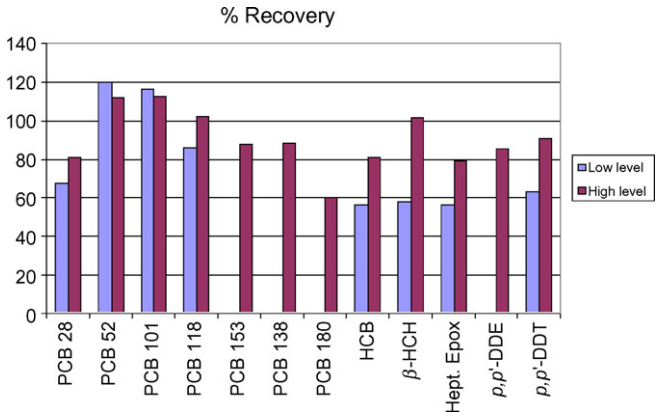

Fig. 2. Mean percent recovery of method ($n = 8$ per level) for selected PCBs and OCPs calculated from fortified human serum at two concentration levels (see text). Low level calculations for congeners 138, 153, 180 and *p,p′*-DDE were not done because serum background levels were far over fortification low levels.

tific). The oven program was started at 65 °C held for 1.5 min, programmed at 20 °C/min to 180 °C held for 9 min, then programmed at 10 °C/min to 230 °C held for 7 min, and finally programmed at 3 °C/min to 275 °C and held 7 min. The MS interface was held on a temperature of 300 °C, the ion source (EI, 70 eV) temperature was 230 °C and the quadrupole temperature 150 °C. The MS detector was operated in the selected ion monitoring (SIM) mode with a dwell time of 40 ms per ion. For quantification and identification the masses shown in Table 1 are used. As identification criteria a relative percent uncertainty of less than ±20% from the theoretical relative abundance of the qualifier ion was considered acceptable.

2.4. Analytical procedure

Serum samples, 500 μl each, were put into 2 ml vials. Then 500 μl of Na_2SO_4 5% water solution and 250 μl of internal standard spiking solution containing PCBs 46 and 143 at 5 ng/ml in acetonitrile, were added to serum. Vials, sealed with screw caps and Teflon-faced silicone septa, were placed in an ultrasonic

Table 2
Limit of quantification (LOQ) of selected polychlorinated biphenyls and organochlorine pesticides in serum

Compound	LOQ (ng/ml)
PCB 28	0.10
PCB 52	0.13
PCB 101	0.10
PCB 118	0.10
PCB 138	0.10
PCB 153	0.08
PCB 180	0.08
p,p′-DDT	0.40
p,p′-DDE	0.33
Heptachlor epoxide	0.32
HCB	0.16
β-HCH	0.38

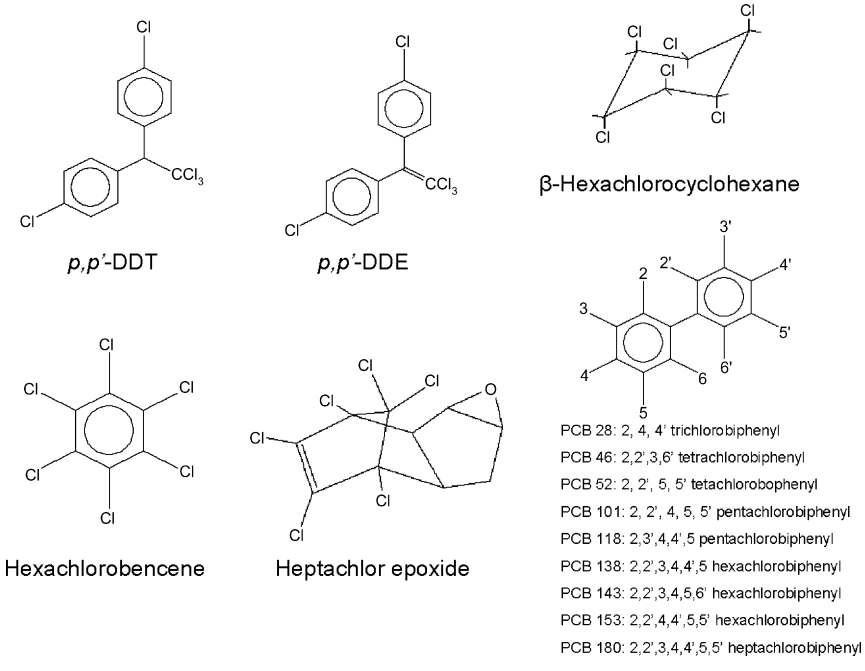

Fig. 1. Chemical structures for target analytes and ISs.

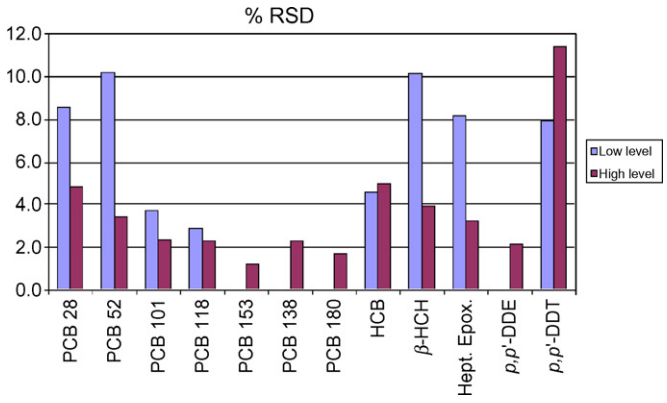

Fig. 3. Short term precision of method for selected PCBs and OCPs, expressed as %R.S.D. ($n = 8$ per level) and calculated from fortified human serum at two concentration levels (see text). Low level calculations for congeners 138, 153, 180 and *p*,*p*′-DDE were not done because serum background levels were far over fortification low levels.

bath for 20 min. Meanwhile, 96-well plate was placed on the vacuum manifold and C_{18} disks were conditioned successively with 2×300 μl of dichloromethane, 300 μl of methanol and 2×300 μl of water. Once disks were conditioned, vial contents were loaded onto wells and gentle suction was applied by means of vacuum or N_2 positive pressure when necessary. After sample elution, wells were rinsed with 2×500 μl of water. Then, disks were dried with N_2 at 200 kPa and by centrifugation (15 min, $1500 \times g$). Ninety-six-glass tube recollection rack was put in the vacuum manifold and each one of disks were eluted with 2×300 μl of hexane and 300 μl of dichloromethane/hexane (1:1), collecting the 900 μl into the same glass tube.

For every disk a glass column was filled, from bottom to top, with glass wool, 300 mg of freshly prepared silica-sulfuric acid mixture and 100 mg of Na_2SO_4, and conditioned with 2 ml of dichloromethane, 2 ml of dichloromethane/hexane (1:1) and 2 ml of hexane. The 900 μl of extract were applied to the column and POCs and PCBs were eluted with 2 ml of dichloromethane. After adding 500 μl of cyclohexane, the eluate was concentrated in the TurboVap (25 kPa of N_2, 30 °C) to 50 μl and transferred to a vial for GC analysis.

2.5. Calibration and validation

GC-ECD calibration was performed using standard solutions of the analytes at 0.75, 2, 10 and 50 ng/ml in cyclohexane (*p*,*p*′-DDE 1.5, 4, 20 and 100 ng/ml). For GC-MSD calibration, 2, 10 and 50 ng/ml standard solutions were used. Quantification was carried out using PCB 46 (for HCB, β-HCH, heptachlor epoxide, PCB 28 and PCB52) and PCB 143 (for *p*,*p*′-DDE, *p*,*p*′-DDT, PCB 101, PCB 118, PCB 138, PCB 153 and PCB 180) as internal standards (IS). The ratios between peak areas of analytes and the respective ISs were plotted against the corresponding concentration ratios using inverse square of concentration-weighted regressions.

Short term precision and recovery were determined by spiking LS pooled serum at two levels: 0.15 and 1.5 ng/ml for PCBs and 0.3 and 3.0 ng/ml for OCPs ($n = 8$ per level).

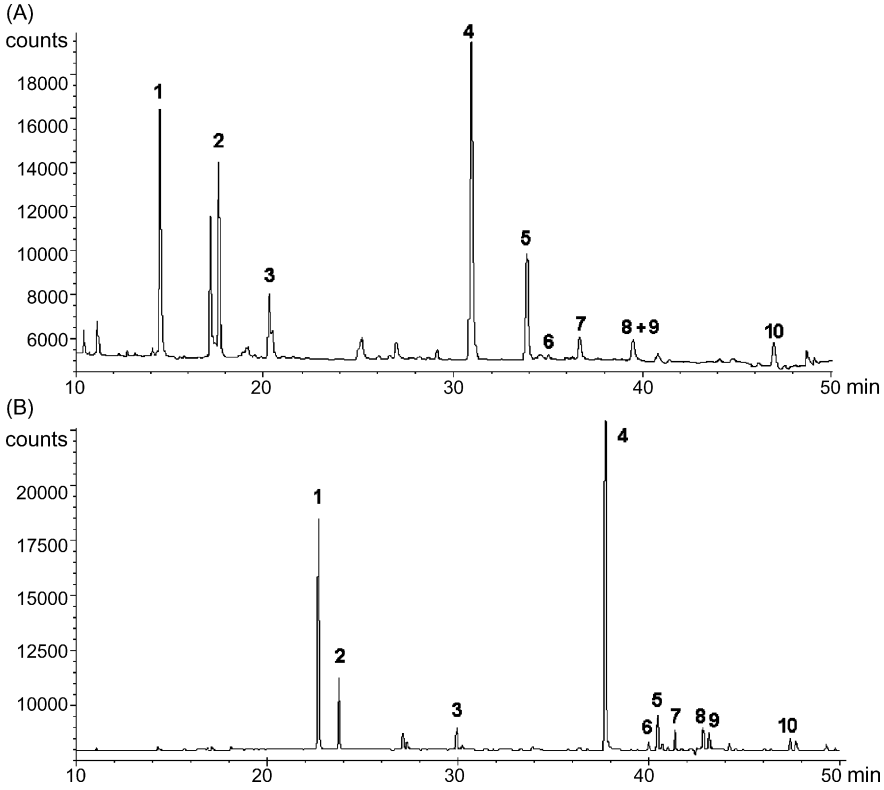

Fig. 4. GC-ECD chromatograms of human serum samples on a DB-XLB column (A) and DB-5 MS column (B). Peak assignment: (**1**) HCB; (**2**) β-HCH; (**3**) PCB 46 (IS); (**4**) *p*,*p*′-DDE; (**5**) PCB 143 (IS); (**6**) PCB 118; (**7**) PCB 153; (**8**) *p*,*p*′-DDT; (**9**) PCB 138; (**10**) PCB 180.

Each sample batch included two procedural blanks and a duplicate of the LS pooled serum (control sample). As procedural blank, 500 μl of water were used instead of serum. The results of the LS pooled serum were displayed in a control chart for every analyte. These results were collected over a yearlong period and used to determine long term precision.

3. Results and discussion

3.1. Method optimization

In order to develop an adequate method using 96-well plates for PCBs and OCPs determination in serum, it is necessary to consider and optimize several parameters that affect the extraction and clean-up procedures.

The choice of denaturant is crucial, because it is responsible for destroying protein–analyte interactions and disrupting micellar formation, both of which decrease recoveries of analytes. On the other hand, if the denaturant is too vigorous could cause excessive protein precipitation, which will clog the extraction disk and result in the loss of the sample or may substantially decrease recoveries because of coprecipitation of the analytes. Several denaturants were tested for optimization of recoveries. Formic acid has been a popular choice of denaturant for extraction [24–26]. In our case formic acid gave good recoveries for PCBs but failed with some OCPs, specially β-HCH and heptachlor epoxide ($R < 50\%$). Trichloroacetic acid and surfactants (Tween 20 and 80, Triton X-100) were also tested. Both gave rise to strong chromatographic contamination. In experiments with Carrez solutions poor recoveries were obtained. Methanol caused excessive protein precipitation, which clogged the extraction disks. The best results were achieved with acetonitrile and 5% anhydrous sodium sulfate solution, because provided the necessary denaturation without causing too much protein precipitation.

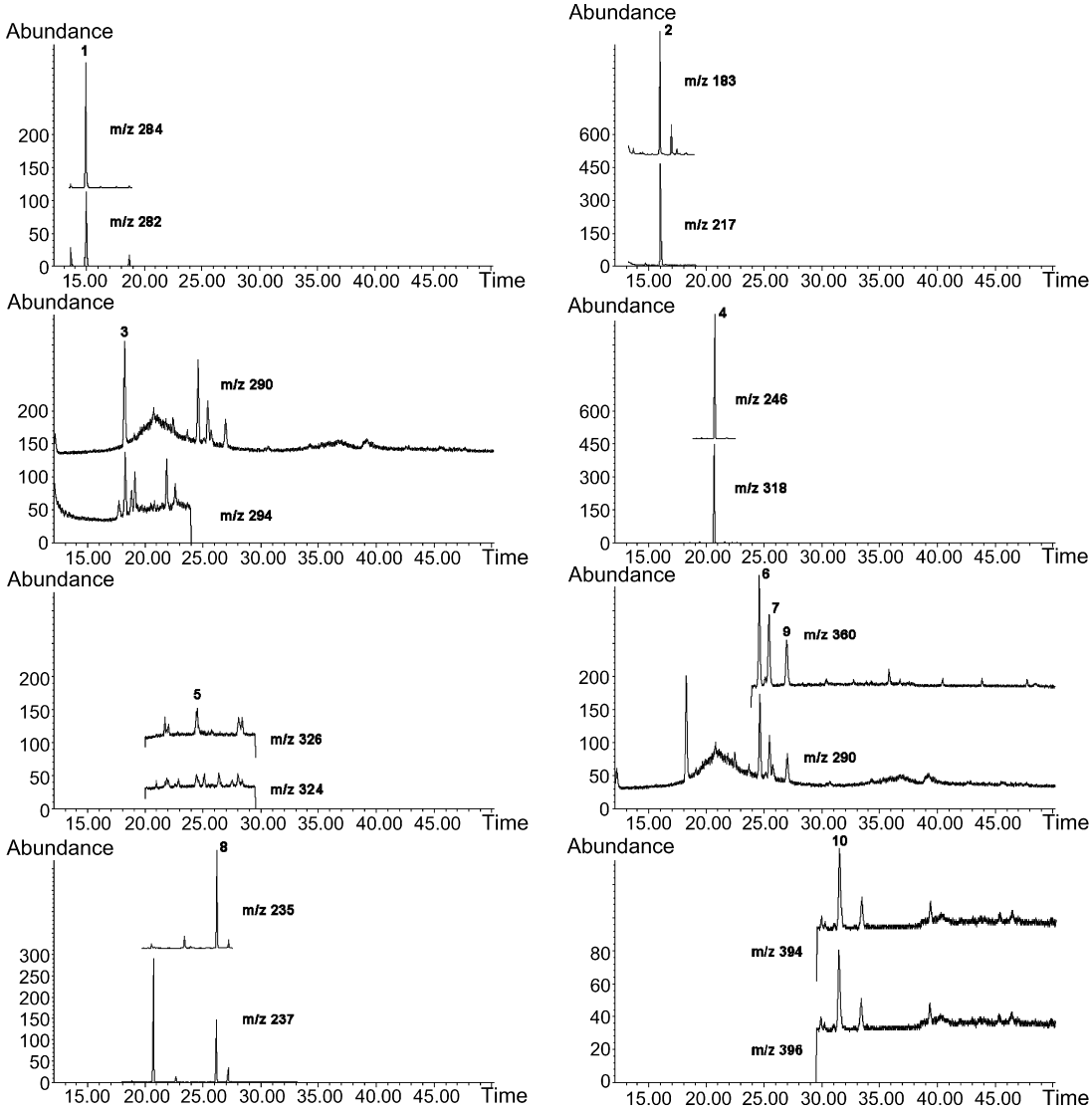

Fig. 5. GC–MS SIM chromatogram of human serum on a DB-5MS column. Peak assignment: (**1**) HCB; (**2**) β-HCH; (**3**) PCB 46 (IS); (**4**) *p,p′*-DDE; (**5**) PCB 143 (IS); (**6**) PCB 118; (**7**) PCB 153; (**8**) *p,p′*-DDT; (**9**) PCB 138; (**10**) PCB 180.

Adsorption columns or concentrated sulfuric acid are been widely used to remove lipids and other co-extractives from non-polar extracts. Recently, several authors described the use of silica/sulfuric acid mixed columns eluted with hexane, and related promising results [21,27]. In experiments with concentrated sulfuric acid we founded that heptachlor epoxide, *o*,*p*′-DDT, α-endosulfan, aldrin, endrin, dieldrin and methoxychlor are sensitive to this acid and degrade. In order to minimize this degradation, elution time must be as short as possible. The reduction of elution time can be accomplished by employing less volume of more polar solvent and shorter clean-up columns. With this aim dichloromethane was tested and we found that 2 ml were enough to complete elution without altering clean-up performance. In the same way, columns with different quantities of silica/sulfuric acid (200, 300, 400 and 500 mg) were also studied to optimize column size. Three hundred milligrams of column yielded good recoveries and did not jeopardize clean-up result.

3.2. Method evaluation

After establishing the extraction and clean-up conditions, the method was checked respect to limits of quantification, recovery and short term precision.

The limit of quantification (LOQ) of each compound was calculated as the concentration of the analyte that produced a signal 9 times higher than the baseline noise of a procedural blank. The LS pooled serum was not used as a "blank" since the sample contained several of the target OCPs and PCBs. LOQ for PCBs ranged from 0.06 to 0.16 ng/ml and for OCPs from 0.16 to 0.61 ng/ml.

For recovery and short term precision evaluation, only five OCPs (HCB, β-HCH, Heptachlor epoxide, *p*,*p*′-DDE, *p*,*p*′-DDT) and seven PCBs (28, 52, 101, 118, 138, 153, 180) were used (chemical structures in Fig. 1). On the basis of our experience these were that could be expected in samples at levels above set LOQ. Fig. 2 shows recoveries of the 12 compounds. Short term precision for each of them, measured as relative standard deviation, is presented in Fig. 3 and LOQ is given in Table 2.

3.3. Application to serum samples

The analytical procedure obtained in this work was applied to 1000 human serum samples of an epidemiological study whose results will be object of another article. The list of compounds founded in these samples is shown in Table 3.

Table 3
Compounds above LOQ in the serum samples of the epidemiological study

Compound	Samples above LOQ (%)
PCB 118	99.9
PCB 138	100
PCB 153	100
PCB 180	100
p,*p*′-DDT	26.4
p,*p*′-DDE	98.0
HCB	89.4
β-HCH	76.5

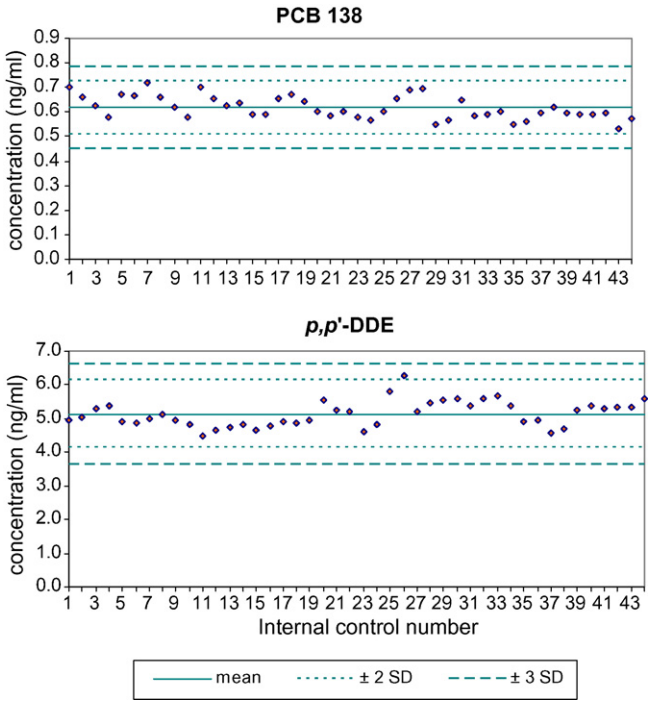

Fig. 6. PCB 138 and *p*,*p*′-DDE control charts. Concentrations measured over a yearlong period ($n = 44$).

GC-ECD representative chromatograms of serum samples on DB-XLB and DB-5MS columns are presented in Fig. 4. As it can be seen, peak resolution was adequate for the target analytes quantification with the exception of *p*,*p*′-DDT and PCB 138 that co-eluted on DB-XLB column and could be corrected using alternative DB-5MS column. GC–MS SIM chromatogram of a serum sample is given in Fig. 5.

Processing batch size was 48–96 serums. Preparative stage took one analyst working day. Each sample batch included a duplicate of the LS pooled serum as internal control. The results of these controls for PCBs 118, 138, 153 and 180, HCB, β HCH, and *p*,*p*′-DDE concentrations were monitored in control charts (Fig. 6) over a yearlong period. Evaluation of long term precision from these results, measured as relative standard deviation, give values <15% for all of them.

4. Conclusions

The 96-well SPE disk plate procedure developed in this paper allows the simultaneous determination of significant OCPs and PCBs in human serum. Compared with other techniques the validated method presents several advantages that make it very suitable for exposition risk or epidemiological studies, where analysis of large number of samples is required. These advantages are: acceptable LOQ with low sample volume (500 μl) as well as good recovery and precision, and improved throughput (48–96 samples per day can be prepared by one analyst without special automatic equipment). It was successfully applied to the analysis of 1000 serum samples from different places of Spain. Moreover, this method could be enlarged to include

other contaminants because non-specific extraction sorbents and conditions are used.

Acknowledgments

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