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Improved sample preparation method for selected persistent organochlorine pollutants in human serum using solid-phase disk extraction with gas chromatographic analysis

A. Pauwels[a,*], D.A. Wells[b], A. Covaci[a], P.J.C. Schepens[a]

[a]*University of Antwerp (UIA), Toxicological Center (Pharmaceutical Sciences), Universiteitsplein 1, B-2610 Wilrijk, Belgium*
[b]*3M Company, Bioanalytical Research & Development, St. Paul, MN 55144 USA*

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Abstract

An improved solid-phase extraction (SPE) method was developed to isolate and concentrate trace levels of selected POPs (persistent organochlorine pollutants) in human serum prior to GC–MS in SIM mode or GC–ECD quantitation. The extraction involves denaturation of serum proteins with formic acid, SPE using C_{18} Empore™ disk cartridges, followed by elimination of lipid interferences using a sulfuric acid wash of the eluate. Use of the SPE disk improved assay throughput and gave a cleaner analytical matrix compared with previously reported solid-phase and liquid–liquid extraction techniques. The extraction method provided consistent recoveries at three fortification levels using $^{13}C_{12}$ PCB 149 as internal standard. Recoveries ranged from 48 to 140% for organochlorine pesticides (6.25, 12.5 and 25 ng/ml) and 71 to 126% for polychlorinated biphenyls (0.625, 1.25 and 2.5 ng/ml).

Keywords: Serum; Solid-phase extraction; Organochlorine pesticides; Polychlorinated biphenyls

1. Introduction

Organochlorine pesticides (OCPs) are effective pest control agents which have been used in agriculture worldwide. Polychlorinated biphenyls (PCBs) are another group of chemicals which have found widespread use in a number of applications, and are present in products such as heat-transfer fluids, hydraulic fluids, capacitors and transformers. These two groups of selected POPs (persistent organochlorine pollutants), each contain numerous isomers and analogs. They have been shown to be ubiquitous environmental pollutants due to their great chemical stability and lipid solubility. POPs are routinely detected in fish, wildlife, human adipose tissue, blood and breast milk [1,2].

The group of pesticides selected for this study were chosen based on their occurrence, persistence, toxicity, and prevalence in human matrices [3,4]. The group consists of β-HCH, γ-HCH (lindane), DDT and its metabolites, heptachlor epoxide, and dieldrin. Specific PCB congener analysis has become

*Corresponding author: Fax: +32-3-820-2734; e-mail: apauwels@uia.ua.ac.be

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the preferred method of monitoring PCBs in the environment [5,6]. Many European government and regulatory bodies have selected seven PCB congeners (numbering according to Ballschmiter et al. [7]: IUPAC Nos. 28, 52, 101, 118, 138, 153, 180) as marker compounds to monitor occurrence and distribution. These congeners were also analyzed in this study.

The development of a fast, simple, sensitive and efficient sample preparation method prior to analytical detection for a range of POPs in serum is important for assessment studies that document health issues related to human exposure. The numerous reported liquid–liquid extraction (LLE) methods for separating organochlorine pesticides and PCBs from human body fluids, and quantifying them at low concentrations, are complex and labor intensive [3]. Generally, these methods involve liquid–liquid extraction of plasma or serum using hexane, hexane/diethyl ether or hexane/acetone [8–13].

Solid-phase extraction (SPE), using reversed-phase C_{18} bonded silica, has been investigated as a superior method of extraction for POPs from serum [3,14–16]. These SPE methods, compared with LLE, offer the advantages of simplicity, reduced solvent usage, and higher throughput. However, most packed column methods for SPE use 100 to 500 mg sorbent mass loadings for 1 to 10 ml serum, thus requiring 5 to 10 ml solvent volumes to complete the extraction steps.

The present paper describes the use of SPE disk technology, instead of traditional packed column technology, to provide a simplified and improved sample preparation procedure for the extraction of some selected POPs from serum, with analysis by GC–MS or GC–ECD. The small bed volume disk approach to SPE reduces elution volumes [17,18]. The final extract from disk extraction is cleaner than packed column SPE. The sample preparation method involves disruption of serum protein-binding using formic acid, then extraction and concentration of analytes using C_{18} solid-phase extraction disk cartridges. Further clean-up of lipid interferences is accomplished using a sulfuric acid wash of the eluate. Quantification is achieved using capillary gas chromatography with mass spectrometry in SIM (selected ion monitoring) mode or with electron capture detector.

2. Experimental

2.1. Sample collection

Sampling of patients was conducted in collaboration with the University Hospital of Antwerp (UZA, Edegem, Belgium). Different batches of human serum were obtained from the Blood Transfusion Center. Blood was collected in a vacuum system tube, transported in a cooling pail, and centrifuged (15 min, 2000 *g*) within 24 h after collection. The serum was pooled and kept frozen at −20°C until analyzed.

2.2. Equipment and reagents

Empore extraction disk cartridges (C_{18}, 7 mm/3 ml and 10 mm/6 ml) and a Varian Positive Pressure Manifold (Part 1223-420X) were provided as gifts from 3M Company (St. Paul, MN, USA). Acetonitrile, dichloromethane (DCM), ethylacetate, *n*-hexane, and methanol were pesticide-grade (CRB, Dublin, Ireland); formic acid, sulfuric acid and triethylamine (TEA) were analytical grade (Merck, Darmstadt, FRG). Reference pesticide standards included all compounds under investigation and were purchased in crystalline form from J.T.Baker (Deventer, The Netherlands). The seven marker PCBs were purchased as a standard mixture from J.T.Baker at a concentration of 10 ng/μl in iso-octane. The $^{13}C_{12}$-labeled chlorinated biphenyls used as surrogate analyte and internal standard (PCB 110 and 149, respectively) were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). A standard mixture solution containing all analytes under investigation was prepared in *n*-hexane.

2.3. Extraction and clean-up

The SPE cartridges were conditioned before sample loading by washing with 250 μl dichloromethane and allowing the column to dry thoroughly. The column was then conditioned with 250 μl of methanol followed by 500 μl deionized water. The cartridge was not allowed to dry before sample application. A 1.0-ml aliquot of serum sample was mixed in a tube with standard mixture solution and $^{13}C_{12}$ PCB 110 as surrogate analyte. Added to the tube were

1 ml formic acid, 50 μl acetonitrile, and 10 μl TEA. Samples were equilibrated by ultrasonic treatment for 30 min. The mixture was loaded onto the SPE disk and gentle suction was applied with positive pressure (2–5 p.s.i.).

After serum loading, the cartridge was rinsed with 500 μl deionized water. The sorbent bed was then thoroughly dried by centrifugation (15 min, 2000 *g*). Analytes were eluted sequentially with two volumes of 200 μl ethylacetate: hexane: TEA (80:20:0.2, v/v), followed by two volumes of 200 μl ethylacetate–hexane–TEA (20:80:0.2, v/v). The eluates were combined, concentrated to 400 μl and mixed with 300 μl concentrated sulfuric acid. After separation of the phases by centrifugation (5 min, 2000 *g*), the organic layer was removed. The sulfuric acid layer was washed again with 300 μl hexane. The organic layers were combined and 100 μl water was added. After centrifugation, the organic layer was transferred to an autosampler vial and concentrated under a gentle stream of nitrogen at room temperature to approximately 20 μl. Temperatures higher than 30°C caused loss of OCPs with low b.p. and PCBs having a low degree of chlorination. The internal standard $^{13}C_{12}$ PCB 149 was added to the final concentrate, prior to GC analysis. The SPE scheme is shown in Table 1.

The method of extraction has been further evaluated by analyzing 8 ml aliquots of human serum using a larger 10mm/6ml disk cartridge size (the 1.0 ml serum aliquots were extracted using a 7mm/3ml disk cartridge). To avoid overloading of the 10mm/6ml cartridges, two columns in parallel, each containing 4 ml of sample, were used. $^{13}C_{12}$ PCB 110 was added as surrogate analyte. The mean surrogate analyte recovery was 97.5±4.9% ($n=15$).

2.4. Chromatographic equipment and analytical conditions

A Hewlett-Packard 5890 series II *Plus* gas chromatograph (HP, Palo Alto, CA, USA) was equipped with a HP 5972A quadrupole mass spec-

Table 1
Ions (m/z) selected in the GC–MS determination of organochlorine compounds and their experimental retention times (t_R)

Compound		Quantifier ion (m/z)	Qualifier ions (m/z)	Experimental t_R (min)
Organochlorine pesticides				
β-HCH		183	181, 217	13.21
γ-HCH		183	181, 217	13.99
Heptachlorepoxide		253	135, 183	18.78
Dieldrin		263	108, 277	22.15
o,p′-DDT		235	165, 237	23.99
p,p′-DDT		235	165, 237	27.03
o,p′-DDD		235	165, 237	22.09
p,p′-DDD		235	165, 237	24.88
o,p′-DDE		246	176, 318	19.42
p,p′-DDE		246	176, 318	21.50
Polychlorinated biphenyls				
Chlorine substitution pattern[a]	**Congener IUPAC No**[b]			
2,4,4′	PCB-28	258	186, 256	14.87
2,2′,5,5′	PCB-52	290	220, 292	15.85
2,2′,4,5,5′	PCB-101	328	256, 326	19.89
2,3′,4,4′,5	PCB-118	328	256, 326	24.13
2,2′,3,4,4′,5	PCB-138	360	290, 358	25.09
2,2′,4,4′,5,5′	PCB-153	360	290, 358	27.12
2,2′,3,4,4′,5,5′	PCB-180	394	324, 392	31.05

[a] Numbering indicates level and position of chlorine substitution.
[b] Numbering system according to Ballschmiter et al. [7].

trometric detector and a fused-silica DB-XLB capillary column (J&W Scientific, Folsom, CA USA) of 60 m×0.25 mm I.D.×0.25 μm film thickness. The GC temperature program was as follows: initial 65°C held for 2 min, then to 220°C at a rate of 50°C min^{-1}, 1.5°C min^{-1} to 255°C, and then 20°C min^{-1} to 290°C, held for 5 min. The pressure pulse program consisted of an initial pressure of 20 p.s.i. held for 0.5 min, 90 p.s.i. min^{-1} to 36 p.s.i., kept for 1 min, 90 p.s.i. min^{-1} to 20 p.s.i., and then 0.54 psi min^{-1} to 36 p.s.i., kept for 2 min. The transfer liner was kept at 280°C. Carrier gas was helium (N56 grade, Air Liquide, Liège, Belgium) at a constant flow of 1.1 ml. A volume of 2 μl was injected splitless at an injector temperature of 260°C.

The mass spectrometer was operated in the electron impact (EI) ionization mode at 70 eV. The mass spectra of individual compounds were determined by injecting 1 μl of each standard (15 ng/μl) into the GC–MS programmed to scan ions from m/z 50 to 500. A SIM table was set up for GC–MS quantitation. Two ions in each molecular ion cluster were monitored. The confirmation of PCBs was accomplished on M^+ and $[M+2]^+$ clusters of ions. For the PCB congeners at least one ion in the $(M-70)^+$ ion cluster was present. Ions (m/z) selected in the GC–MS determination of POPs and OCCs and corresponding retention times are shown in Table 1. The retention time, the masses and the relative abundance of the confirmation ion to the quantitation ion were used as the identification criteria. A deviation of the isotope ratio of less than ±20% from the theoretical value was considered acceptable but usually the deviation was within ±10%. For each m/z value, the dwell time was 100 ms and the delay time was 10 ms.

To measure background concentrations of unspiked serum samples, a Hewlett-Packard 6890 Series gas chromatograph (HP, Palo Alto, CA USA) with electronic pneumatics control (EPC) was equipped with an autosampler, and HP 6890 Micro-ECD system. Carrier gas was hydrogen at a constant pressure of 135 kPa. A volume of 2 μl was injected splitless at an injector temperature of 260°C. The μ-ECD temperature was kept at 320°C, make-up gas, argon–methane (95+5) at a flow-rate of 20 ml/min. The GC column and the temperature program were equal as described before.

Table 2
Method detection limits (MDL) and limits of quantification(LOQ) for every compound under investigation (pg/g)

Compounds	MDL	LOQ
Organochlorine pesticides		
β-HCH	270	900
γ-HCH	350	1170
o,p′-DDT	300	1000
p,p′-DDT	340	1130
o,p′-DDD	160	530
p,p′-DDD	300	630
o,p′-DDE	170	570
p,p′-DDE	240	800
Polychlorinated biphenyls		
PCB 28	122	407
PCB 52	97	323
PCB 101	64	213
PCB 118	51	270
PCB 138	92	307
PCB 153	61	203
PCB 180	36	120

2.5. Calibration and detection limits

Multi-level calibration curves were created for the quantification using standard solutions of the analytes in hexane. Quantification was carried out using $^{13}C_{12}$ PCB 149 as internal standard. Method detection limits (MDL) at $S/N=3$, as well as the limits of quantification (LOQ) at $S/N=10$, were determined for every compound using the above mentioned analytical conditions. Detection limits obtained using this procedure are summarized in Table 2.

3. Results and discussion

3.1. Protein binding

Dale et al. [8,9] used hexane for the extraction of chlorinated insecticides from blood but found that the recoveries were not quantitative, due to binding of the analytes to serum proteins. Pretreatment of the serum by denaturation was found to liberate the compounds from protein binding sites [11,12]. The relative efficiency of protein denaturation methods was evaluated without precipitation because of the possible loss of analytes by occlusion in the precipi-

tate [19]. Experiments with acid denaturation methods (formic acid, trichloroacetic acid, perchloric acid), organic solvents (methanol, acetonitrile) and zinc salt in alkaline solution were performed. The formic acid deproteination technique yielded the highest recoveries for the individual POPs examined in this study (Table 3).

3.2. SPE conditions

Initial solid-phase extractions of serum spiked with OCPs and PCBs did not yield adequate recovery, as analytes were protein bound and did not adsorb to the reversed-phase C_{18} silica upon sample loading. Higher yields were obtained after optimization of the sample application, washing and elution steps. It has been reported that addition of detergents or triethylamine (TEA) during sample application masks some of the strong hydrophobic or polar sites from exposed silanol groups, resulting in higher recoveries [20]. The addition of the detergent Triton X-100 to the serum did not improve recoveries for these analytes. However, higher yields were obtained by applying 0.1% TEA to the serum samples before protein denaturation, and also by adding 0.2% TEA to the eluting solvent (Table 3).

In the washing step, the choice of solvents is restricted to polar aqueous solvents because a decrease in solvent polarity causes co-elution of analytes [21]. Water alone was preferred as the washing solvent because the use of even small percentages (10%) of methanol or acetonitrile in water caused some elution of analytes. The use of slower flow-rates (2–5 p.s.i. required) allowed for maximal residence time of the solvents with the sorbent bed and yielded slightly improved recoveries than higher flow-rates (10–15 p.s.i. required).

The elution pattern chosen, consisting of two times 200 μl of ethylacetate–hexane–TEA (80:20:0.2, v/v), followed by two times 200 μl of ethylacetate–hexane–TEA (20:80:0.2, v/v), yielded higher recoveries for these analytes than any pure solvent tested (hexane, ethylacetate, dichloromethane). The use of two sequential aliquot volumes was preferred as it also offered slightly improved recoveries compared with a single elution aliquot.

3.3. Sulfuric acid clean up

Removal of lipids from nonpolar extracts can be achieved by washing with concentrated sulfuric acid or by passing the solvent through adsorption columns of florisil, aluminium oxide or silica gel. The latter method does not provide complete removal of lipids, because the polarity of fats is similar to those of pesticides [22], thus yielding interferences during the chromatographic run. After analyses of a few serum samples extracted using C_{18} extraction columns, without a subsequent sulfuric acid adsorption step, the GC chromatogram indicated that contamination was present. This contamination was removed when eluates were cleaned with concentrated sulfuric acid, demonstrating the benefit of this acid treatment. Moreover, Vacutainer™ tubes and closures for serum

Table 3
Optimization steps in extraction development

	Pesticides		PCB		Experimental conditions[a]
	Mean	Range	Mean	Range	
1	47	(23–57)	53	(28–104)	C_8
2	41	(21–58)	39	(27–50)	Equilibration 3 h
3	41	(30–65)	38	(18–63)	Fast elution (20 p.s.i.)
4	8	(6–13)	6	(4–7)	1 ml serum + 1 ml MeOH: supernatant over SPE
5	19	(12–28)	16	(10–19)	1 ml serum + 1 ml acetic acid
6	78	(52–117)	79	(40–144)	1 ml serum + 1 ml formic acid
7	73	(37–95)	78	(60–87)	1 ml serum + 1 ml formic acid + 5% acetonitrile
8	75	(37–99)	84	(66–95)	1 ml serum + 1 ml formic acid + 0.2% TEA
9	83	(51–127)	81	(46–111)	1 ml serum + 1 ml formic acid + 5% acetonitrile + 0.2% TEA
10	82	(50–127)	95	(77–113)	Optimal conditions: C_{18} + equilibration 30 min + slow elution (2 p.s.i.): 1 ml serum + 1 ml formic acid + 5% acetonitrile + 0.2% TEA

[a] $n = 6$ for each optimization step.

storage bottles are suspected sources of phthalate ester contamination [10]. During the clean-up of the extracts in the adsorption columns, the phthalates were eluted in the same fraction as the analytes. Peaks from phthalates overlapped some of the analytes under investigation. The clean-up of extracts with concentrated sulfuric acid also removed the ubiquitous phthalate esters, thus eliminating the interferences in the GC chromatogram and giving a reliable determination of the analytes under investigation [22]. Note that heptachlor epoxide and dieldrin are degraded during clean-up with concentrated sulfuric acid.

3.4. Analyte recovery

Determination of recoveries was performed using pooled human serum spiked with different amounts of analytes. The recovery rates for each fortified serum sample were determined by adding the internal standard $^{13}C_{12}$ PCB 149 to the sample extract just before GC–MS analysis. A mean recovery of 85.0±6.6% of $^{13}C_{12}$ PCB 110, the compound added to all samples as a surrogate analyte, was considered satisfactory in our protocol. The fortification levels corresponded to three specific levels for each pesticide and PCB (25, 12.5 and 6.25 ng/g for pesticides and 2.5, 1.25 and 0.625 ng/g for each PCB). Recoveries of eight pesticides and seven PCBs spiked into human serum were consistent for each analyte, yielding recoveries of 48–140% and 71–126%, respectively, for pesticides and PCBs. Complete recovery information is shown in Table 4.

Table 4
Percentage recoveries of 15 compounds from fortified human serum (three fortification levels[a])

Compounds	High level[b] % *R* (RSD)	Med. level[c] % *R* (RSD)	Low level[d] % *R* (RSD)
Pesticides			
β-HCH	55 (19)	48 (19)	48 (7)
γ-HCH	64 (8)	interf.[f]	interf.[f]
o,p′-DDT	76 (7)	70 (20)	84 (6)
p,p′-DDT	72 (9)	77 (27)	76 (16)
o,p′-DDD	77 (9)	64 (7)	72 (7)
p,p′-DDD	76 (6)	69 (4)	68 (14)
o,p′-DDE	88 (10)	94 (9)	94 (11)
p,p′-DDE	93 (7)	92 (4)	140 (6)
PCBs			
PCB-28	104 (11)	123 (15)	111 (13)
PCB-52	104 (9)	120 (12)	113 (13)
PCB-101	85 (8)	85 (5)	97 (4)
PCB-118	74 (7)	85 (18)	88 (9)
PCB-138	77 (11)	98 (24)	126 (12)
PCB-153	71 (14)	99 (26)	114 (19)
PCB-180	72 (25)	83 (23)	77 (10)
$^{13}C_{12}$ PCB-110[e]	90 (7)	77 (5)	88 (8)

[a] $n=6$ at each fortification level.
[b] Compounds spiked at 25 ng/g (pesticides) and 2.5 ng/g (PCBs) into pooled human serum.
[c] Compounds spiked at 12.5 ng/g (pesticides) and 1.25 ng/g (PCBs) into pooled human serum.
[d] Compounds spiked at 6.25 ng/g (pesticides) and 625 pg/g (PCBs) into pooled human serum.
[e] Surrogate analyte.
[f] Interferences at low fortification levels.

3.5. Human serum samples

A typical capillary GC–MS separation of the eluate from an 8-ml unfortified serum sample, after C_{18} solid-phase extraction, is shown in Fig. 1. The chromatogram shows background amounts of the metabolite *p,p*-DDE, as well as some PCB congeners. The chromatogram of this blank sample can be compared with a 1-ml serum extract, spiked at the highest fortification level (Fig. 2).

Based on LOQ of the mass spectrometric detector and on concentrations of the analytes in literature reports, all human serum samples were also analyzed by GC–ECD. The background levels obtained (Table 5) are in agreement with literature values of serum concentrations in the Netherlands and Sweden [2,23].

4. Conclusions

Solid-phase extraction (SPE) using C_{18} Empore disk cartridges was successfully applied for the determination of some POPs from human serum, after denaturation of these analytes from serum proteins. SPE was preferred over liquid–liquid techniques for its smaller solvent volumes and ease of use. The SPE disk allowed for further reductions in solvent volumes (compared with traditional packed columns), which were ideal for ultratrace determinations. Quantification of analytes was achieved by GC–MS in SIM mode and GC–ECD. The SPE method was shown to be consistent at three fortifica-

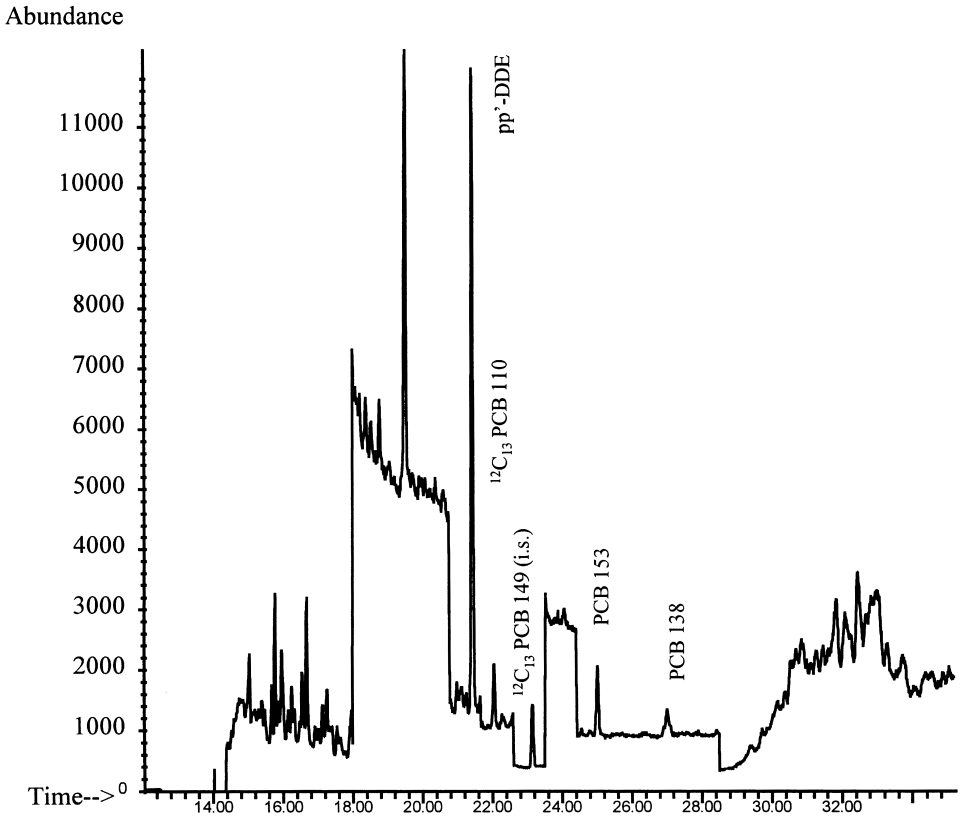

Fig. 1. GC–MS chromatogram of 8-ml unfortified serum sample.

Table 5
Background levels of POPs in blank human serum (pg/g)

	This study ($n=15$)		The Netherlands[a] ($n=415$)		Sweden[b] ($n=9$)	
	mean	range	Mean	Range	Mean	Range
β-HCH	625	(319–882)				
γ-HCH	703	(352–899)				
o,p′-DDT	194	(76–440)				
o,p′-DDE	808	(201–2351)				
p,p′-DDT	430	(211–706)			60	(40–120)
p,p′-DDD	644	(165–1282)				
p,p′-DDE	4657	(1116–9896)	N.D.[c]		2400	600–5100)
PCB 118	142	(63–349)	160	(20–600)	120	(50–210)
PCB 138	450	(236–757)	600	(130–1600)	N.D.	
PCB 153	404	(146–798)	910	(180–2500)	1500	(600–3500)
PCB 180	243	(127–456)	540	(80–3100)	1000	

[a] The Netherlands 1994 [23].
[b] Sweden 1994 [2].
[c] N.D.: not detected.

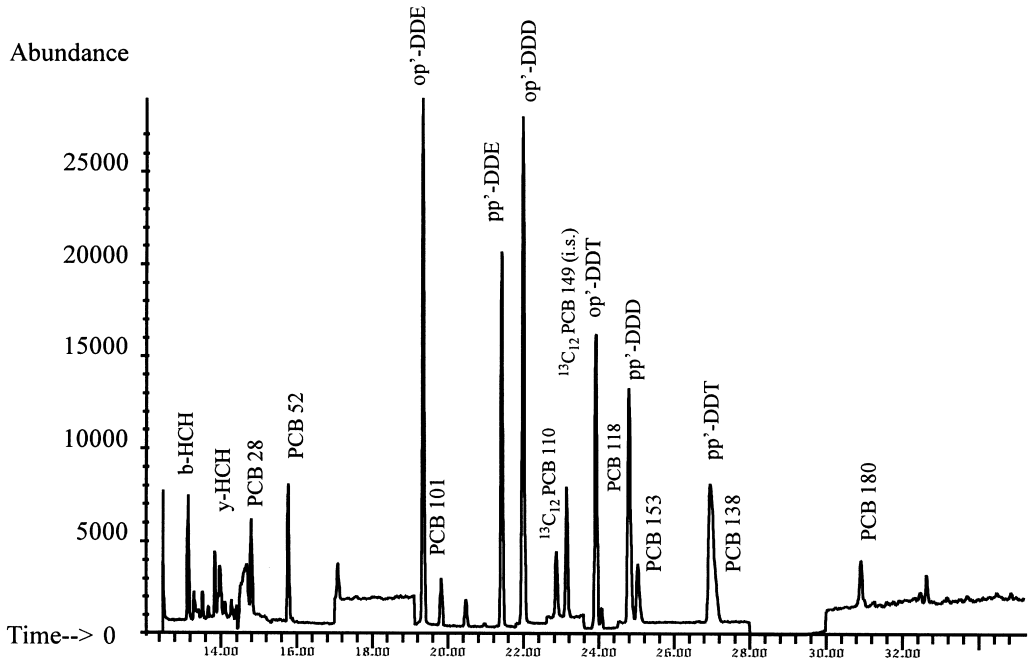

Fig. 2. GC–MS chromatogram of 1-ml spiked serum sample (fortification level: 25 ng/g for pesticides and 2.5 ng/g for PCBs).

tion levels, providing recoveries of 48–140% and 71–126% for pesticides and PCBs, respectively.

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