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Determination of polychlorinated biphenyls in small-size serum samples by solid-phase extraction followed by gas chromatography with micro-electron-capture detection ☆

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Abstract

An new method for the determination of polychlorinated biphenyls (PCBs) in serum samples of up to 1 ml has been developed. The procedure consisted in the solid-phase extraction (SPE) of the analytes on an Oasis cartridge and the subsequent on-line elimination of the fat by directly dropping of the eluate from the SPE cartridge on a multilayer column placed below the cartridge. This configuration allowed minimising of the sample manipulation as well as the time, solvent and sorbent consumption (i.e. complete sample preparation can be accomplished in about 1 h with only 3 ml of toluene and 300 mg of silica). The SPE plus clean-up method developed showed a satisfactory performance for the analysis of PCBs in rat serum samples providing similar recoveries (i.e. range 73–128% for most of the congeners selected) at the different spiking levels investigated (1.25, 0.50 and 0.25 ng/ml). Detection limits using a microelectron capture detector were in the range 0.01–0.30 ng/ml of serum and the relative standard deviations of the complete method better than 18% irrespective of the PCB concentration. The validated method has been applied to the evaluation for the first time of the PCB levels in serum samples of up to 1 ml from individuals of an Egyptian Vulture colony in Spain.

Keywords: Micro-electron-capture detection; Polychlorinated biphenyls

1. Introduction

Polychlorinated biphenyls (PCBs) are a group of ubiquitous pollutants that are usually present in environmental samples as complex mixtures [1]. Among the 209 possible isomers, attention is usually focused on those congeners that show similar toxicity to that of polychlorodibenzo-*p*-dioxins and dibenzofurans [2]. Due to their wide use in the past and their persistence, measurable levels of PCBs can nowadays be found worldwide. Nevertheless, the lipophilicity of these compounds and their ability to be bioconcentrated by organisms makes the species at the highest positions in food chains to be among the most affected by their presence in the environment. This is so in the case of predatory birds such

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as Egyptian Vulture (*Neophron percnopterus*), a species in danger of extinction. The relevance of this species makes the preservation and control of its scarce colonies of main concern. That control should include the evaluation of the levels of pollutants such as PCBs in the individuals. In this kind of monitoring study, the analysis of blood or serum samples can be regarded as one of the more plausible approaches to determine the actual healthiness of the individuals. However, the relatively small amount of blood sample available (typically up to 1 ml of serum) adds an extra difficulty to this type of study.

Most of the previously reported methods for the analysis of PCBs in serum, plasma or blood are based on tedious and time consuming liquid–liquid extraction procedures [3,4] that usually require volumes of sample larger than 10 ml [3–5]. Some alternative methods based on the solid-phase preconcentration of the samples on sorbents such C_{18} [5,6] or carbon [7] have been developed in an attempt to simplify the sample preparation and simultaneously reduce the solvent and time consumption. However, analytical procedures for PCBs determination in serum or blood samples as small as 1 ml are still scarce in the literature [6,8].

The goal of this study was to develop a new analytical method based on the solid-phase extraction (SPE) of PCBs from serum samples of up to 1 ml by using a relatively new SPE sorbent, Oasis (Waters). Clean-up of the extracts was carried out by direct elution of the extraction solvent from the SPE cartridge through a multilayer column as this approach effectively contributed to reducing the total solvent consumption and the sample manipulation. Gas chromatography with microelectron capture detection (GC–micro-ECD) was used for final determination of the target compounds. The developed method has been applied to the evaluation of the PCB levels in serum samples collected in an Egyptian Vulture colony in Spain.

2. Materials and methods

2.1. Chemicals

All solvents used were pestipur quality and were purchased from SDS (Peypin, France), except hexane (Merck, Darmstadt, Germany) and acetonitrile (Fisher, Pittsburgh, PA, USA). Formic and sulphuric acids were pro analysis quality (Merck). Anhydrous sodium sulphate was obtained from J.T. Baker (Deventer, The Netherlands) and silica gel 60 from Merck. Extraction cartridges of Oasis HLB (60 mg, Waters, Milford, USA) were used for sample enrichment.

The 23 PCB congeners studied (see Table 1) were selected because of their toxicity and relative abundance in environmental samples [2]. A working stock solution was prepared from individual PCB standards (Ehrenstorfer, Augsburg, Germany) containing 1000 pg/μl of each compound in isooctane. This solution was used for further dilution and spiking of the samples in the preliminary experiments. 1,2,3,4-Tetrachloronaphtalene (TCN, Ehrenstorfer) and PCB 209 were used as external standards for PCB quantification (by calculating the relative response factors of the analytes against TCN) and identification (by calculating the relative retention times against PCB 209) during the GC–micro-ECD determination.

Serum of laboratory rats was used for the preliminary experiments and the optimisation of the analytical method. Rat blood was collected in glass tubes, kept at 4°C for 30 min and then centrifuged (Microfuge 11, Beckman, CA, USA) at 13000 rpm for 10 min to separate the serum. Blood samples of birds were taken from a population of Egyptian Vulture (*Neophron percnopterus*) in Fuerteventura (Spain) and processed as described to yield serum samples up to 1 ml. All samples were conserved at −20°C until analysed.

2.2. Procedure

In all experiments, the SPE cartridges were placed in an SPE-12G (J.T. Baker) apparatus attached to a water aspirator via a pressure-metering valve and conditioned with 2 ml of dichloromethane, 2 ml of methanol and 2 ml of Milli-Q water (Millipore, Bedford, USA) before using.

Some preliminary experiments were carried out to optimise the SPE step. In this part of the study, Milli-Q water spiked with the appropriated amount of the target compounds dissolved in acetone and equilibrated for 15 min in an ultrasonic bath was

Table 1
Analytical data for the GC–micro-ECD analysis of standard solutions

PCB no. [9]	Linear range (ng/ml)	Regression coefficient[a]	Repeatability (RSD, %)[b]	Reproducibility (RSD, %)[c]
28	0.5[d]–60	0.9992	8	40
52	0.5–60	0.9991	20	31
95	0.5–75	0.9999	7	10
101	0.5–100[e]	0.9998	7	9
77	0.5–100	0.99998	5	16
123+149	0.5–200	0.9999	4	15
118	0.5–100	0.9997	5	15
114	0.5–100	0.9998	5	17
153	0.5–100	0.9995	4	13
105+132	0.5–200	0.9995	4	16
138	0.5–100	0.9998	8	16
126	0.5–100	0.9997	7	16
183	0.5–100	0.9998	7	15
167	0.5–100	0.9997	4	15
156	0.5–100	0.9995	2	17
157	0.5–100	0.9997	2	17
180	0.5–100	0.9998	5	16
169	0.5–100	0.99998	3	13
170	0.5–100	0.9997	3	16
189	0.5–100	0.9998	1	16
194	0.5–100	0.99996	2	14

[a] For response vs. area plot.
[b] As calculated at the 10 ng/ml level ($n=3$).
[c] As calculated at the 10 ng/ml level ($n=3$).
[d] Minimum concentration level included in the calibration line.
[e] Maximum concentration level included in the calibration line.

used. Three levels of concentration (2.50, 1.25 and 0.50 ng/ml of each PCB) were tested. A method previously published for the analysis of PCBs in human serum with C_{18} disk cartridges [6] has been now adapted for PCB determination using Oasis SPE cartridges. Briefly, a 1-ml aliquot of the corresponding sample was mixed with 1 ml of formic acid and 50 μl of acetonitrile and equilibrated in an ultrasonic bath for 20 min. The mixture was allowed to pass through the SPE cartridge, which was then washed with 1 ml of Milli-Q water and subsequently dried for 20 min under suction. The analytes were eluted with three times 1 ml of toluene at a flow-rate of around 0.5 ml/min and collected in microvials.

Once the SPE step was optimised with Milli-Q water samples, some additional experiments were carried out for further validation of the whole SPE plus clean-up procedure developed for serum analysis. In this part of the study, pooled rat serum samples spiked with the target compounds and equilibrated for 15 min in an ultrasonic bath were used. Three levels of concentration (1.25, 0.50 and 0.25 ng/ml of each PCB) were tested. Serum samples were prepared as previously described but the 3 ml eluent from the SPE cartridge was directly eluted through a second multilayer column containing 60 mg of silica gel activated at 140°C for 48 h, 240 mg of silica modified with sulphuric acid (44%, w/w) and anhydrous sodium sulphate [10]. Fig. 1 shows a schematic diagram of the arrangement proposed. The toluene eluting from this second column was collected in microvials for subsequent GC–micro-ECD analysis.

In all cases, the final extracts were concentrated under a gentle stream of nitrogen. The external standard solution containing TCN and PCB 209 was added to vials prior to GC–micro-ECD analysis. Unless otherwise specified, experiments were carried out in triplicate.

Experiments were carried out in a fume hood to

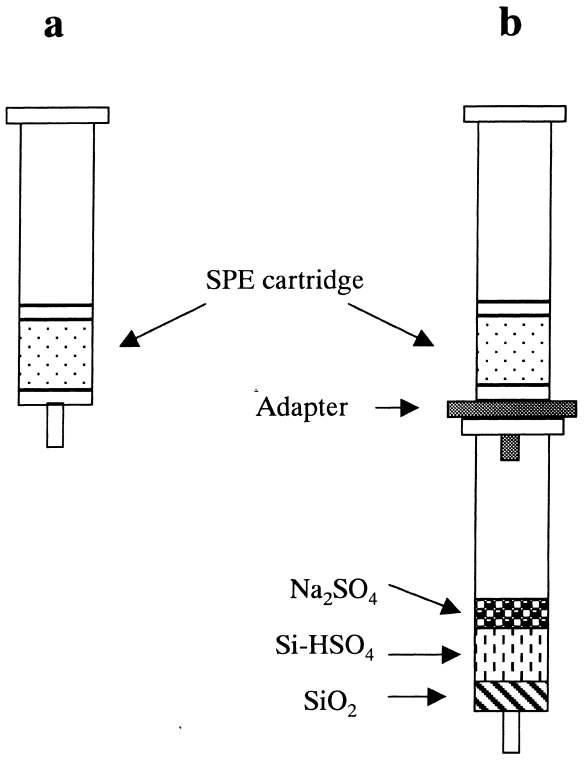

Fig. 1. Schematic diagram of the arrangement proposed for (a) SPE preconcentration of the analytes and (b) desorption of the target compounds from the SPE cartridge and simultaneous clean-up of the fatty extracts.

prevent from any possible contamination. Blank samples were analysed to check any contamination throughout the analytical procedure. Milli-Q water and serum blanks were prepared following the same procedure as in water and rat serum experiments, respectively, but without spiking. No background interference was found to be introduced by the methodology proposed. In the recovery experiments, if levels of endogenous PCBs detected were above the quantification limit, they were subtracted from the spiked serum samples results to allow realistic estimation of the method performance.

Finally, the SPE plus clean-up method developed was applied to the determination of the endogenous levels of the PCBs selected in 13 Egyptian Vulture serum samples.

2.3. Instrumentation

PCB determination in the final extracts was performed by GC (HP 6890 Series, Hewlett-Packard, Palo Alto, CA, USA) with micro-ECD. Samples were injected in the hot splitless mode (1 μl, 270°C, splitless time 1.0 min) in a capillary DB-5 column (60 m×0.25 mm I.D., 0.25 μm film thickness) purchased from J&W Scientific (USA). The column temperature was programmed from 80°C (2 min) to 185°C (3 min) at a rate of 30°C/min, then to 230°C (15 min) at 1.5°C/min and then to 270°C (15 min) at 5°C/min. Nitrogen was used as carrier gas (constant flow, 1.5 ml/min) and as make-up gas 30 ml/min. The detector temperature was set at 300°C.

Confirmation of the presence and relative abundance of the individual PCB congeners investigated was carried out in a GC (CP-3800, Varian, CA, USA) equipped with an ion trap MS detector (Saturn 2000, Varian) working in the selected ion monitoring (SIM) mode. Samples were injected in the hot splitless mode (1 μl, 250°C, splitless time 1.0 min) in a capillary BPX-5 column (60 m×0.25 mm I.D., 0.25 μm film thickness) purchased from SGE (Australia). The column temperature was programmed from 140°C (1 min) to 200°C (1 min) at a rate of 20°C/min and then to 300°C (10 min) at 3°C/min. Helium was used as the carrier gas at a constant head pressure of 47 p.s.i. (1 p.s.i.=6894.76 Pa). The temperature of the transfer line and the trap were set at 300 and 250°C, respectively. For each compound, the two most abundant ions produced by electron ionisation (EI) at 70 eV were monitored [11].

3. Results and discussion

3.1. GC–micro-ECD analysis

Table 1 summarises the results obtained when the linearity, repeatability and ruggedness of the micro-ECD were tested for the PCBs investigated. To evaluate the linearity of the detector response, standard solutions containing the 23 PCBs were prepared. For PCBs 28 and 52 and for PCB 95 responses were linear over the range 0.5–60 ng/ml and 0.5–75 ng/ml, respectively, with regression

coefficients better than 0.9991 ($n=6$). For all other PCBs, regression coefficients were better than 0.9995 ($n=7$) over a concentration range greater than three orders of magnitude (range tested 0.5–100 ng/ml). The repeatability, which was determined by analysing a standard solution at the 10 ng/ml level, was satisfactory with relative standard deviations (RSDs) lower than 8%, except for PCB 52 (20%). The reproducibility, which was determined by analysing a standard solution at the 10 ng/ml level randomly inserted in the real-samples series (i.e. each 12 h of operation) within a week, was satisfactory with RSD values of 9–17% for most of the analytes (RSDs of the two early eluting congeners, PCBs 28 and 52, 40% and 31%, respectively). The experimentally determined limits of detection (LODs) were in the range 0.10–0.50 ng/ml in the final extract (i.e. 0.1–0.5 pg injected). All these results were in the range of those previously reported for organochlorine pesticides using micro-ECD [12] and for PCBs with more linear and (usually) sensitive detectors such as MS [6,11]. More importantly, these results showed that reliable quantification should be possible for PCBs at the levels typically encountered in environmental samples even if the initial amount of sample used for the analysis is reduced. The wider linear range of the micro-ECD, which contributed to reduce the sample handling in PCB analysis (i.e. dilution or concentration and reanalysis of the final extracts) was considered an additional advantage of this new detector.

3.2. Optimisation of the SPE plus clean-up procedure and application

Preliminary experiments were carried out to optimise the SPE step. For this study, water samples spiked at the 2.50, 1.25 and 0.50 ng/ml levels were used.

The feasibility of the Oasis sorbent for the simultaneous determination of a broad range of priority organic pollutants, including some of the PCBs investigated in the present work (IUPAC Nos. 28, 52, 77, 126, 138, 153, 169 and 180) in aqueous samples has been previously evaluated [13]. In that study, 200 ml of water spiked at the 1.00–2.00 ng/ml level were concentrated in an Oasis 60 mg SPE cartridge. Elution of the target compounds with 2.5 ml of acetonitrile–dichloromethane (1:1) plus 3.2 ml of dichloromethane provided recoveries of the eight PCBs analysed in the range 65–84%.

As one typical example of the results obtained in this part of the present study, Table 2 summarises the recoveries found after SPE of 1 ml of the water spiked at the lowest level investigated (i.e. 0.50 ng/ml). (In this table, the compounds investigated have been organised according to their chemical structure, i.e. the planarity and total number of chlorines of the PCB congeners increase from the top to the bottom of the table). Recoveries in the range 85–124% were obtained for all the target compounds (except PCB 189, 68%) with RSDs lower than 17% ($n=3$). These results were similar to those found at the other two spiking levels tested (data not shown). Not unexpectedly, these recovery values were somewhat higher than those previously obtained with the quoted above screening method involving Oasis SPE cartridges [13]. The higher selectivity of the extraction solvent selected in the present study, toluene, for PCBs could be suggested as a possible explanation for the observed differences. On the other hand, the reduction achieved in the volume of organic solvent required for quantitative elution of the analytes (3 ml in this study vs. 5.7 ml) due to the use of toluene was considered as an additional merit of the methodology developed. Finally, it is important to note that although the polarizability of the π-electron cloud could be suggested as a possible key factor governing the retention of the PCBs on this particular sorbent, under the experimental conditions proposed (data not shown), no discrimination problems were observed for the isomers studied (Table 2).

Once the performance of the SPE step was proved for spiked water, rat serum samples were used to further proceed with method validation. Among the different procedures reported for fat removal in PCB analysis, sulphuric acid treatment have been usually preferred to the use of sorbents such florisil [14], alumina [5] or carbon [15] because of the limited capacity for fat retention of the latter. In this study, a multilayer column containing silica and silica modified with sulphuric acid (44%, w/w) [10] was used for fat removal and clean-up of the SPE extracts as

Table 2
Chemical structure of the PCBs investigated, number of chlorines in *ortho*-position and analytical performance of the SPE method developed for PCB determination in water (spiking level, 0.50 ng/ml)

PCB no.	Structure	No. of *ortho*-Cl	Recovery (RSD)
95	22′35′6	3	112 (9)
132[a]	22′33′46′	3	113 (9)
149[b]	22′34′5′6	3	94 (17)
183	22′344′5′6	3	96 (4)
52	22′55′	2	104 (11)
101	22′455′	2	91 (8)
138	22′344′5′	2	124 (13)
153	22′44′55′	2	96 (11)
170	22′33′44′5	2	94 (14)
180	22′344′55′	2	93 (13)
194	22′33′44′55′	2	91 (11)
28	244′	1	73 (15)
118	23′44′5	1	104 (4)
123[b]	2′344′5	1	–
114	2344′5	1	85 (8)
105[a]	233′44′	1	–
167	23′44′55′	1	107 (12)
156	233′44′5	1	123 (4)
157	233′44′5′	1	117 (5)
189	233′44′55′	1	68 (5)
77	33′44′	0	128 (7)
126	33′44′5	0	110 (6)
169	33′44′55′	0	107 (8)

Recoveries and RSDs (%) were calculated from three separate analyses.
[a] PCBs 105 and 132 coeluted under the GC experimental conditions used in the study.
[b] PCBs 123 and149 coeluted under the GC experimental conditions used in the study.

this approach was cleaner, faster and involved a smaller amount of organic solvents and acid than those methods based on direct addition of sulphuric acid to either the fatty extract [3,6] or the SPE cartridge containing the analytes [7]. This multilayer column was placed down the SPE cartridge and directly couple to it. This configuration allowed to minimise the total solvent consumption as the solvent extracting the PCBs from the SPE cartridge was also used for the elution of the analytes through the clean-up column. The consequent reduction of the time required for sample preparation (e.g. by eliminating the concentration step of the fatty extracts previous to its application to the multilayer column) and the minimisation of the risk of contamination by reducing the sample manipulation were considered additional benefits of this configuration.

The analytical performance of the complete SPE plus clean-up procedure proposed for the determination of the PCBs selected in real-life sample was evaluated by analysing rat serum samples spiked at three levels of concentration (1.25, 0.50 and 0.25 ng/ml). Three separate analyses were carried out for each of the three spiking levels. Relevant analytical data are summarised in Table 3. The total procedure showed a satisfactory performance for the analysis of PCBs in serum at the three spiking levels studied with recoveries laying within the 73–128% range for most of the congeners. Lower values, i.e. 51–63%, were only found for the more chlorinated planar congeners investigated, PCBs 157, 189 and, especially, 169. This result could be related to a higher retention of these compounds on the SPE sorbent due to the relatively easy polarization of their π-electron cloud, a phenomenon also observed in sorbents such florisil [8,16] and, especially, carbon [7]. Due to the toxicological significance of PCB 169, the relatively low recovery obtained in the present study for this particular congener could make recommend the use of labelled internal standard for its determination in

Table 3
Analytical performance of the complete SPE plus clean-up method developed for PCB determination in serum at three levels of spiking

PCB no.	Recovery (RSD)			LOD
Spiking level (ng/ml)	1.25	0.50	0.25	
3-*ortho*-Cl				
95	124 (4)	92 (10)	109 (5)	0.18
132 (+105)	87 (4)	110 (3)	118 (5)	0.22
149 (+123)	124 (1)	91 (1)	94 (8)	0.04
183	92 (11)	84 (8)	75 (13)	0.01
2-*ortho*-Cl				
52	78 (6)	108 (12)	125 (3)	0.14
101	96 (1)	124 (3)	117 (10)	0.19
138	91 (12)	125 (11)	101 (7)	0.08
153	106 (1)	114 (6)	108 (14)	0.19
170	102 (9)	77 (1)	63 (12)	0.04
180	96 (12)	99 (6)	88 (6)	0.25
194	79 (2)	88 (3)	82 (18)	0.08
1-*ortho*-Cl				
28	118 (4)	93 (14)	88 (15)	0.18
118	122 (5)	101 (1)	95 (6)	0.05
114	130 (4)	76 (1)	80 (10)	0.08
167	101 (11)	90 (8)	73 (5)	0.02
156	95 (7)	90 (4)	74 (8)	0.05
157	85 (5)	62 (5)	51 (2)	0.03
189	95 (7)	62 (10)	71 (14)	0.20
0-*ortho*-Cl				
77	122 (13)	128 (11)	103 (14)	0.10
126	115 (11)	114 (9)	98 (13)	0.04
169	60 (1)	56 (9)	56 (9)	0.30

Recoveries and RSD values (%) were calculated from three separate analyses. LOD (ng/ml of serum) as experimentally determined (S/N, 3:1) in serum samples.

real-life samples. However, this approach would make the use of GC–MS for final determination mandatory. The RSD values, which were essentially the same irrespective of the PCB concentration level, were below 18% for all PCBs. These results were similar to those reported for C_{18} SPE cartridges (in the range 71–126%) [6] and substantially better than those published for SPE Florisil columns (in the range 2–60%) [8], both methods involving larger amounts of sample and solvents and higher spiking levels than those used in the present study. The results obtained with the method developed were also in the range of those obtained with tedious and solvent consuming LLE methods (globally 44–147% with RSD in the range 6–31% at a spiking level of 0.50 ng/ml) [4,15]. The LODs calculated in real rat serum samples (globally in the range 0.01–0.30 ng/ml of serum, Table 3) showed that reliable quantification of the target compounds should be possible at levels that are typically encountered in this matrix [5,6] even if only 1 ml of sample is used for the analysis and contributed to support the practicability of the analytical method developed for PCB determination in small size serum samples.

The validated SPE plus clean-up method was finally applied to the analysis of the endogenous PCB levels in 13 samples of serum of up to 1 ml from individuals of a colony of Egyptian Vulture in Spain. Table 4 summarises the results obtained for this set of samples. The reported PCB concentrations have not been corrected by the recoveries obtained for the corresponding spiked congeners in the previous experiments involving rat serum. The PCB levels in the serum analysed ranged from 0.03 to 27.1

Table 4
Concentrations of the endogenous PCBs (ng/ml of serum) in the bird serum samples analysed

PCB no.	Geometric mean ($n=13$)	SD	Range
95	0.91	0.73	0.28–3.04
132+105	7.32	6.80	1.83–27.1
149+123	0.26	0.29	ND–0.86
183	0.11	0.44	ND–1.58
52	0.60	0.63	0.18–2.35
101	1.29	1.78	0.52–7.27
138	1.72	2.70	0.40–10.6
153	2.65	4.21	0.66–16.5
170	0.44	2.21	ND–8.13
180	2.02	3.43	0.47–11.5
194	0.37	0.54	0.03–1.72
28	0.61	0.45	0.24–1.50
118	0.37	0.60	0.04–2.39
114	ND[c]	–	–
167	0.11	0.25	ND–0.87
156	0.11	0.13	ND–0.44
157	0.11	0.29	ND–1.05
189	0.52	0.40	ND–1.43
77[a]+110	0.58	0.77	0.03–2.98
126[b]+129+178	0.17	0.15	ND–0.43
169	ND	–	–

[a] Coelution with PCB 110 cannot be ruled out.
[b] Coelution with PCBs 129 and 178 cannot be ruled out.
[c] Not detected.

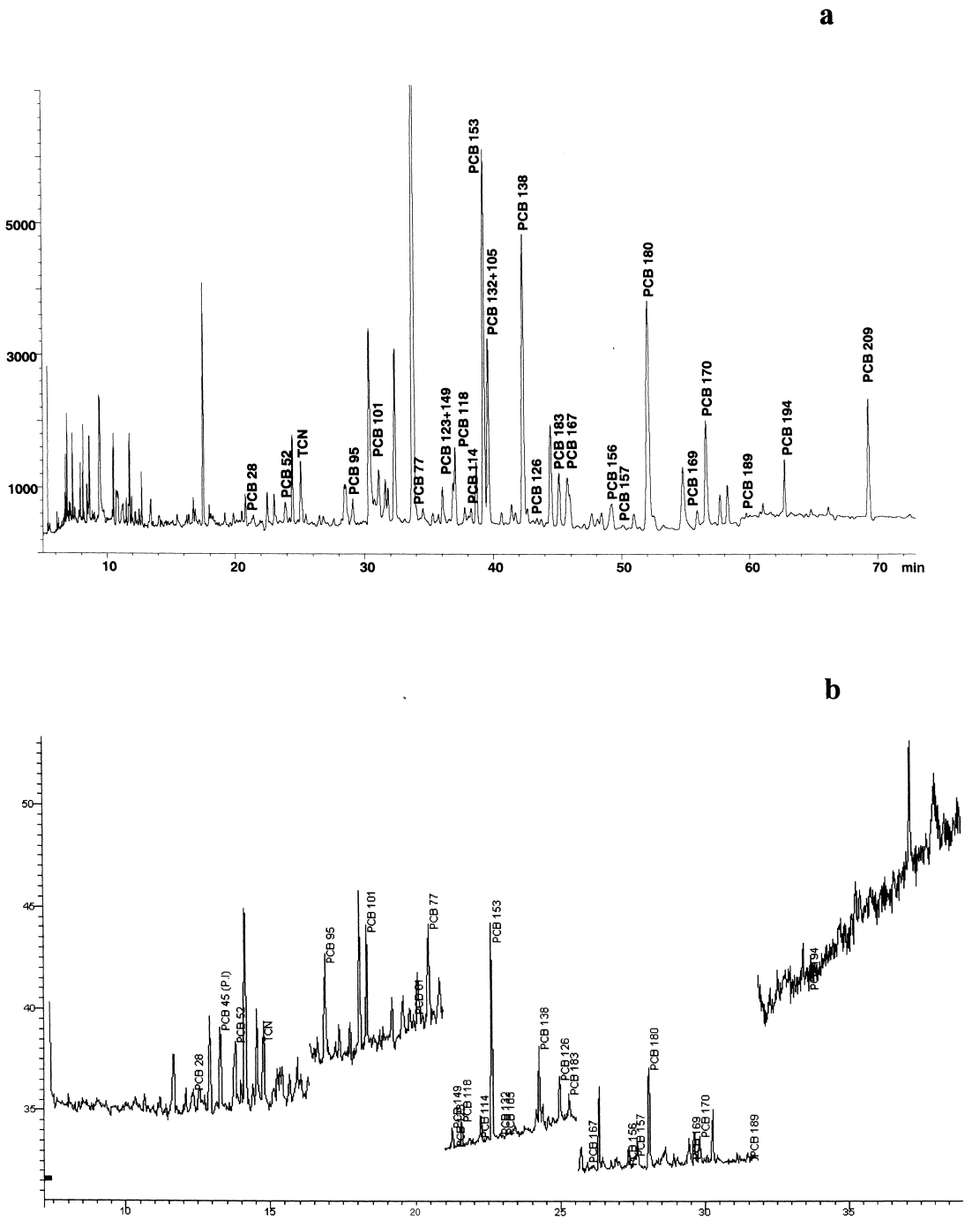

Fig. 2. Typical PCB chromatograms obtained for a non-spiked bird serum sample by (a) GC–micro-ECD and (b) GC–MS (ITD/SIM) after SPE plus clean-up of the analytes according to the method developed.

ng/ml. As expected, PCBs 101, 132(+105) and 153 were the most abundant congeners among those studied. Nevertheless, the standard deviations (SDs) calculated for these PCBs were also among the highest (in the 6.80–1.73 range). The most toxic compounds (i.e. PCBs 77, 126 and 169) were usually found to be at levels below the LOD once the interferences affecting their quantification by GC–micro-ECD were solved using a GC–MS. (Note that values reported in Table 4 correspond to concentrations as calculated by GC–micro-ECD). No previous data related to the PCB levels in serum of this species were found in the literature. However, rather similar PCB patterns were obtained for all the serum samples of the individuals investigated in this study. As an example of the results obtained, Fig. 2 shows the typical GC–micro-ECD and GC–MS (ITD/SIM) chromatograms obtained for a non-spiked bird serum sample after SPE plus clean-up according to the method developed.

4. Conclusions

The practicability of a fast SPE plus clean-up procedure for the determination of PCBs in small size serum samples has been shown. Based on an optimised SPE of the analytes on an Oasis cartridge, the method allowed a complete sample preparation with only 3 ml of toluene and 300 mg of silica for additional clean-up of the extracts. The procedure can be completed in about 1 h but up to 9 samples can be simultaneously processed. The performance of the procedure was satisfactory irrespective of the PCB concentration and similar to, or better than, that of previously published methods which usually involved larger amounts of samples and organic solvents. The use of the micro-ECD for final determination of the analytes allowed to achieve LODs low enough (i.e. 0.01–0.30 ng/ml of serum) to ensure the accurate quantification of the target compounds even if samples of up to 1 ml were used. As an illustration of the potential of the proposed methodology for monitoring studies, the validated procedure has been applied to the evaluation of the endogenous levels of PCBs in individuals of a protected species of bird (Egyptian Vulture).

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