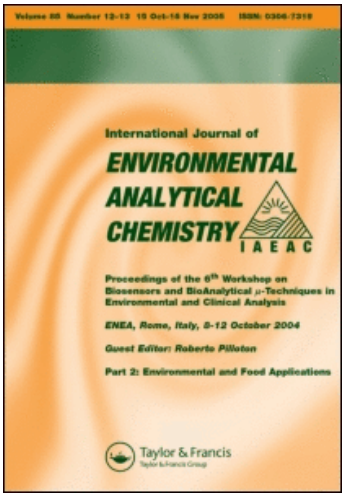

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Determination of Selected Persistent Organochlorine Pollutants in Human Serum by Solid-phase Disk Extraction and Dual-Column Capillary Gas Chromatography with Electron Capture Detection

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DETERMINATION OF SELECTED PERSISTENT ORGANOCHLORINE POLLUTANTS IN HUMAN SERUM BY SOLID-PHASE DISK EXTRACTION AND DUAL-COLUMN CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

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An improved clean-up method by solid-phase disk extraction was developed to isolate and concentrate trace levels of POPs (persistent organochlorine pollutants) in human serum prior to gas chromatography with electron capture detection on two different capillary columns, providing an improved selectivity. An Empore™ C_{18} bonded silica extraction disk cartridge is used for the initial extraction and enrichment of the analytes. Subsequent clean-up is achieved by concentrated sulphuric acid and silica gel adsorption chromatography. Recoveries for selected POPs are ranging from 62 to 74% and a good reproducibility (RSD < 14%) is demonstrated. Human samples analysed under these conditions, show a similar relative concentration profile.

Keywords: PCB; persistent organochlorine pollutants; human serum; solid phase disk extraction; dual column; electron capture detector

INTRODUCTION

Polychlorinated biphenyls (CBs) and organochlorine pesticides are among the most prevalent environmental contaminants. Because of their lipophilicity, and long half-lives, residues of these two groups of persistent organic pollutants (POPs) or their metabolites tend to bioaccumulate in the adipose tissues of biota and humans[1].

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CBs and organochlorine pesticides are of public health concern[2–7]. The requirements for risk assessment in epidemiological studies have created the need for efficient, fast and less-costly analytical methods. Monitoring of human exposure to POPs is most conveniently performed by analysis of the blood plasma or blood serum.

Due to trace levels of POPs in biological fluids and the presence of other extraneous chemicals at higher concentration levels, a highly sensitive and selective multistage analytical procedure is needed. The determination of POPs by high resolution gas chromatography (HRGC) usually requires preliminary purification of the extracts before instrumental analysis in order to improve the process of quantification.

Conventional methods of separating POPs from human body fluids involve liquid-liquid extraction with non-polar solvents[8–13]. They are very complex, labour intensive, time consuming and use excessive amounts of solvents and reagents. Solid-phase extraction, using commercially available columns pre-packed with various stationary phases has been previously investigated as an alternative method for extraction and clean-up[14–20].

In a previous paper[21] we reported as first the use of solid-phase disk extraction (SPDE) technology for the analysis of POPs from human serum. The procedure involves denaturation of serum proteins with formic acid, solid-phase extraction using C_{18} Empore™ disk cartridges, followed by elimination of lipid interferences using a sulphuric acid wash of the eluate. Use of SPDE improved assay throughput and allowed reduced volumes for elution. However, due to residual interferences, identification and quantification was only possible for CB 118, CB 138, CB 153, CB 180, and p,p'-DDE, and the method did not allow to determine minor CB congeners, which contribute substantially to the total dioxin-like toxicity. The background noise becomes lower using an additional clean-up step, thus allowing the determination of minor peaks. Furthermore, a more accurate quantification is achieved using two different capillary columns.

The purpose of the present work is to provide a reliable, simple, rapid and sensitive methodology for the routine congener specific analysis of CBs.

EXPERIMENTAL

Chemicals

Based on reported abundances and toxicities, the following CB congeners (IUPAC numbering) were targeted for analysis : 18, 28, 31, 44, 52, 66, 74, 99, 101, 105,

110, 118, 28, 138, 149, 153, 156, 157, 167, 170, 180, 183, 187, 194, 199. Additionally, we included hexachlorobenzene (HCB), as recent reports[22] suspect HCB to be a dioxin-like compound and p,p'-DDE, as the major DDT metabolite. CB 46 and CB 143 were used as surrogate standards and 1,2,3,4-tetrachloronaphtalene (TCN) as internal standard. The criteria for their selection were the elution characteristics in the GC columns and their absence in human samples.

All solvents (methanol, acetonitrile, hexane, dichloromethane, acetone, iso-octane) were pesticide grade (Merck, Darmstadt, Germany). Formic acid and triethanolamine (Across, Geel, Belgium) and sulphuric acid (Merck) were analytical grade reagents. Individual CB congeners (10 ng/µl in iso-octane) were purchased from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). All solutions were stored at −20°C. Anhydrous sodium sulphate (Merck) for residue analysis was used after heating overnight at 120°C. Solvents were tested for interferences by concentration from 15 ml to 50 µl.

Apparatus and materials

Individual human serum samples and pooled human serum were provided by the Municipal Hospital of Timisoara, Romania and the Blood Transfusion Centre, University Hospital of Antwerp, respectively. Blood was collected in a vacuum system tube, transported in a cooling pail and centrifuged (15 min. 2000g) within 24 hr after collection. The serum was kept frozen at −20°C until analysed.

All glassware were washed with detergent, rinsed with water, soaked for 24 h in chromic acid and rinsed with distilled water, acetone, and hexane. Prior to use, the treated glassware was rinsed with the solvent with which it would be subsequently in contact.

Empore™ extraction disk cartridges (C_{18}, 10mm/6ml) from 3M Company (St. Paul, MN, USA) and a Varian Positive Pressure Manifold (part 1223–420X) were used for solid-phase extraction. Silica gel cartridges (100 mg/l ml) were purchased from Supelco (Bellefonte, PA, USA).

A Hewlett Packard 5890 series II GC with helium (N56 grade, Air Liquide, Belgium) as carrier gas was equipped with an electron capture detector (ECD) using Ar/CH_4(95:5) as make-up gas (55 ml/min) and with a HP 3395 integrator. Two µl were injected in splitless mode with the split outlet opened after 2 min. Injector and detector temperatures were set at 260°C and 300°C, respectively. Two different capillary GC columns were applied: a 50m × 0.25mm, df=0.10 µm, CP-Sil 5/C18 column, with 10% C18 incorporated in the stationary phase (Chrompack, Middelburg, The Netherlands) and a DB-XLB column, 60m × 0.25mm, df=0.25µm (J&W Scientific, Folsom, USA). The temperature program of the CP-Sil 5/C18 column was set to 80°C for 0.5 min and then

increasing the temperature with 15°C/min to 180°C, kept for 5 min and further by 2°C/min to 260°C kept for 10 min. The temperature program of the other column DB-XLB, was starting from 80°C, kept for 0.5 min and then increasing the temperature with 15°C/min to 220°C, stay 5 min and further by 2°C/min to 275°C kept for 25 min. The inlet pressures were 20 psi and 23 psi for the CP-Sil 5/C18 column and the DB-LXB column, respectively.

The samples were analysed on each column, the lowest value for each congener was further considered for calculations.

Multi-level calibration curves ($r^2 > 0.97$) were created for the quantification using standard solutions in iso-octane, covering the entire range of expected values for each congener. In the graphs peak area ratios (POP response / internal standard response) were plotted against the concentrations of POPs. Method limits of detection range between 5 and 10 pg/ml serum (whole weight) using the above mentioned analytical conditions.

Sample preparation and clean-up

Ten ml of human serum were spiked with surrogate standards CB 46 and CB 143 (1.25 ng/ml serum of each). Before extraction, the spiked sample was equilibrated in an ultrasonic bath for 15 min. The serum was then mixed with 10 ml formic acid, 500 µl acetonitrile and 100 µl triethanolamine and was equilibrated by ultrasonic treatment for 30 min.

Prior to the sample application, the Empore™ disk cartridges were washed with two 500 µl portions of hexane and dried thoroughly. Then, each cartridge was activated with 250 µl of methanol followed by two 250 µl portions of deionized water. After conditioning, the cartridges were not allowed to dry. To avoid overloading and breakthrough of the analytes, two cartridges in parallel, each containing 5 ml of sample, were used. After sample loading at a positive pressure of 2–4 psi, each cartridge was rinsed with two 500 µl portions of deionized water. The sorbent bed was dried thoroughly under a nitrogen stream at 20 psi positive pressure (10 min) and by centrifugation (15 min, 2000 g). Each column was eluted with three 500 µl portions of hexane, which were combined.

One ml of concentrated sulphuric acid was added to the combined eluates and mixed for 3 min. After separation of the phases by centrifugation (5 min, 2000 g), the organic layer was removed. The sulphuric acid layer was washed again with 300 µl hexane. The organic layers were combined and concentrated under nitrogen to approximately 100 µl. A silica gel cartridge topped with 100 mg Na_2SO_4 was pre-washed with 2 ml of a mixture of dichloromethane and hexane (1:1, v/v) and 2 ml of hexane. The concentrated sample was applied to the silica gel cartridge. POPs were eluted with 4 ml hexane. After the addition of 50 µl of

iso-octane as a keeper, the final eluate was concentrated under a gentle nitrogen stream at room temperature to approximately 50 µl and transferred to a vial. The tube containing the extract was rinsed with 50 µl iso-octane which was transferred to the vial. Twenty-five µl of internal standard TCN (500 pg/µl in iso-octane) was added to the final concentrate, prior to GC analysis.

Recoveries and quality assurance

In order to test the method performance, recoveries of surrogate standards and of all investigated compounds from 10 ml spiked serum were determined (Table I). Five replicates at one spiking level and three non-spiked replicates from the same batch of pooled serum were analysed. Absolute recoveries of POPs were calculated after subtraction of levels found in the non-spiked replicates from the spiked ones.

TABLE I Recovery of surrogate standards and investigated compounds from 10 ml of spiked human serum from one batch of pooled serum (n=5)

Comp.	*Chlorine substitution pattern*	*RRT**		*Spiking Level*		
		DB-XLB	*CP-Sil5/C18*	*Added (ng/ml)*	*Recovery (%)*	*RSD (%)*
CB 18	2,2',5	0.760	0.684	0.315	67	12
CB 28	2,4,4'	0.872	0.826	1.250	65	10
CB 31	2,4',5	0.865	0.814	0.315	72	5
CB 44	2,2',3,5'	0.975	0.942	0.315	68	8
CB 52	2,2',5,5'	0.928	0.897	1.250	71	11
CB 66	2,3,4,4'	1.101	1.111	0.315	73	8
CB 74	2,4,4',5	1.083	1.096	0.315	71	10
CB 99	2,2',4,4',5	1.157	1.199	0.315	68	6
CB 101	2,2',4,5,5'	1.142	1.178	1.250	69	9
CB 105	2,3,3',4,4'	1.423	1.471	0.250	72	4
CB 110	2,3,3',4',6	1.250	1.274	0.315	67	12
CB 118	2,3',4,4',5	1.342	1.408	0.250	73	14
CB 128	2,2',3,3',4,4'	1.571	1.614	0.625	68	11
CB 138	2,2',3,4,4',5'	1.473	1.546	1.250	70	7
CB 149	2,2',3,4',5',6	1.300	1.347	0.315	72	12
CB 153	2,2',4,4',5,5'	1.383	1.479	1.250	68	10
CB 156	2,3,3',4,4',5	1.676	1.751	0.250	70	4
CB 157	2,3,3',4,4',5'	1.691	1.756	0.315	74	9
CB 167	2,3',4,4',5,5'	1.587	1.693	0.315	71	5
CB 170	2,2',3,3',4,4',5	1.831	1.871	0.625	65	7

Comp.	*Chlorine substitution pattern*	*RRT**		*Spiking Level*		
		DB-XLB	*CP-Sil5/C18*	*Added (ng/ml)*	*Recovery (%)*	*RSD (%)*
CB 180	2,2',3,4,4',5,5'	1.704	1.802	1.250	66	6
CB 183	2,2',3,4,4',5',6	1.525	1.624	0.315	68	10
CB 187	2,2',3,4',5,5',6	1.505	1.599	0.315	70	8
CB 194	2,2',3,3',4,4',5,5'	2.154	2.136	1.250	67	5
CB 199	2,2',3,3',4',5,5',6	1.865	1.893	0.315	65	12
HCB		0.725	0.667	0.625	62	7
p.p'-DDE		1.219	1.242	3.125	70	10
CB 46†	2,2',3,6'	0.914	0.850	1.250	68	8
CB 143†	2,2',3,4,5,6'	1.315	1.363	1.250	65	9

*. – retention times relative to TCN as internal standard (t_R = 27.028 min on CP-Sil5/C18 and t_R = 29.853 min on DB-XLB)
†. – surrogate standards

The following procedures were used to ensure adequate quality. All peak in each chromatogram were manually reviewed for proper integration. The identification of POPs was based on their relative retention times (RRT) to the internal standard. RRT varying by more than 0.02 units from the corresponding standard were considered as not detected (n.d.). A standard solution including all congeners was analysed every sixth injection to correct for variations in chromatographic and instrument performance. Reagent blanks were run for interference. Recoveries of surrogate standards were monitored to ensure their maintenance at acceptable levels. Spike recoveries should range between 60–115% for at least 80% of the analytes and the percent difference must be less than 25% for the spiked matrix in duplicate.

RESULTS AND DISCUSSION

Solid-phase extraction

The SPE method eliminates the elaborate procedures required by traditional methods for total lipid extraction, followed by various treatments to remove lipids. Pre-treatment of the serum by denaturation was found to release the compounds from the protein binding sites. The protein denaturation method without precipitation has been previously evaluated because of the possible loss of analytes by occlusion in the precipitate[21]. Acetonitrile and reduced pH due to formic acid inhibit protein binding and increase the extraction efficiency of the analyte by the C_{18} sorbent.

The C_{18} disk cartridge employed for sample clean-up and analyte enrichment has a non-polar character in order to retain organochlorine and other non-polar compounds. It has also a size exclusion function to eliminate macromolecular interferences (such as serum proteins) in biological extracts. The small bed volume disk approach to SPE reduces elution volume[23], thus making this method more attractive. The use of slower flow rates (at 2–4 psi) allows maximal residence time of the solvents with the sorbent bed and yields slightly improved recoveries than higher flow rates (at 10–15 psi).

The drying step is essential because the non-polar eluent needs to interact with all areas of the sorbent and should not be stopped by residual water trapped in the pores. Centrifugation of the cartridges before elution of POPs, is necessary due to the high compactness of the adsorbent bed, thus difficult to dry completely.

Removal of lipids and other interfering compounds from the SPE eluate can be achieved by using a concentrated sulphuric acid wash. Further clean up to eliminate non-degraded traces of lipids and other polar interferences, is achieved by silica gel column chromatography. Additional sample clean-up on silica gel resulted in a lower background, which facilitated peak identification of minor congeners, providing better instrumental performance and longer column lifetime (traces of sulphuric acid are retained in the silica gel cartridge).

Recovery experiments

Ten ml of human serum were fortified with 2 surrogate standards and all investigated compounds. Recoveries for selected POPs ranged from 62 to 74% and a good reproducibility (RSD < 14%) was demonstrated. There were no differences in recoveries between CB congeners with a low or high degree of chlorination or with respect to the degree of chlorination in *ortho*-position. Results are depicted in Table I. Recoveries (n=5) for surrogate standards were 68 ± 8% for CB 46 and 65 ± 9% for CB 143, and they were considered satisfactory for our protocol.

Reproducibility and GC separation

In order to test the reproducibility of the method, three non-spiked samples from the same batch of pooled human serum were analysed. The reported concentrations (Table II) were obtained from analyses on two different GC columns (see Methods). Relative standard deviations ≤ 18% (for major congeners, at concentration >150 pg/ml) demonstrate a good reproducibility of the method. Only minor CBs exhibited larger variations, which can be attributed to the concentration being close to the limit of detection.

TABLE II Human serum concentrations (pg/ml whole weight) of selected POPs from one batch of pooled serum (3 replicates) and from individual human samples (n=7)

Comp.	*Chlorine substitution pattern*	*Pooled human serum (n=3) Mean (RSD%)*	*Individual human serum (n=7)*	
			Mean	*Range**
CB 18	2,2',5	n.d.	n.d.	–
CB 28	2,4,4'	30 (75)	77	5 – 209
CB 31	2,4',5	n.d.	n.d.	–
CB 44	2,2',3,5'	n.d.	n.d.	–
CB 52	2,2',5,5'	12 (91)	31	5 – 144
CB 66	2,3,4,4'	24 (83)	86	5 – 210
CB 74	2,4,4',5	129 (21)	185	103 – 376
CB 99	2,2'4,4',5	n.d.	46	5 – 88
CB 101	2,2',4,5,5'	20 (80)	28	5 – 62
CB 105	2,3,3',4,4'	60 (25)	94	5 – 202
CB 110	2,3,3',4',6	23 (76)	45	5 – 90
CB 118	2,3',4,4',5	229 (10)	374	206 – 791
CB 128	2,2',3,3',4,4'	69 (23)	143	84 – 303
CB 138	2,2',3,4,4',5'	863 (5)	1399	562 – 3003
CB 149	2,2',3,4',5',6	n.d.	n.d.	–
CB 153	2,2',4,4',5,5'	1152 (2)	1630	682 – 3235
CB 156	2,3,3',4,4',5	152 (18)	259	123 – 433
CB 157	2,3,3',4,4',5'	n.d.	n.d.	–
CB 167	2,3',4,4',5,5'	76 (22)	110	25 – 185
CB 170	2,2'3,3',4,4',5	243 (12)	480	206 – 1388
CB 180	2,2',3,4,4',5,5'	632 (12)	1288	640 – 3465
CB 183	2,2',3,4,4',5',6	93 (10)	161	94 – 391
CB 187	2,2',3,4',5,5',6	217(12)	431	197 – 929
CB 194	2,2',3,3',4,4',5,5'	42 (42)	137	57 – 471
CB 199	2,2',3,3',4',5,5',6	103 (18)	306	207 – 694
HCB		340 (8)	186	116 – 335
p.p'-DDE		7302 (3)	15813	5294 – 33084

*. – Values found under the detection limit were set at 5 pg/ml whole weight

ECD chromatograms of a serum sample analysed on the CP-Sil5/C18 and DB-XLB columns are shown in Figure 1. The separation of individual CBs on each type of GC stationary phase have previously been investigated[24, 25], but none of these two columns is routinely used for CB analysis. When compared to

the most used GC capillary column in CB analysis – DB-5 (5 % phenyl stationary phase) or equivalent – these two columns offer better separation and avoid co-elution of interesting congeners. Retention times relative to TCN are presented in Table II. To our knowledge, no studies have described yet the use of both columns at the same time for biological matrices.

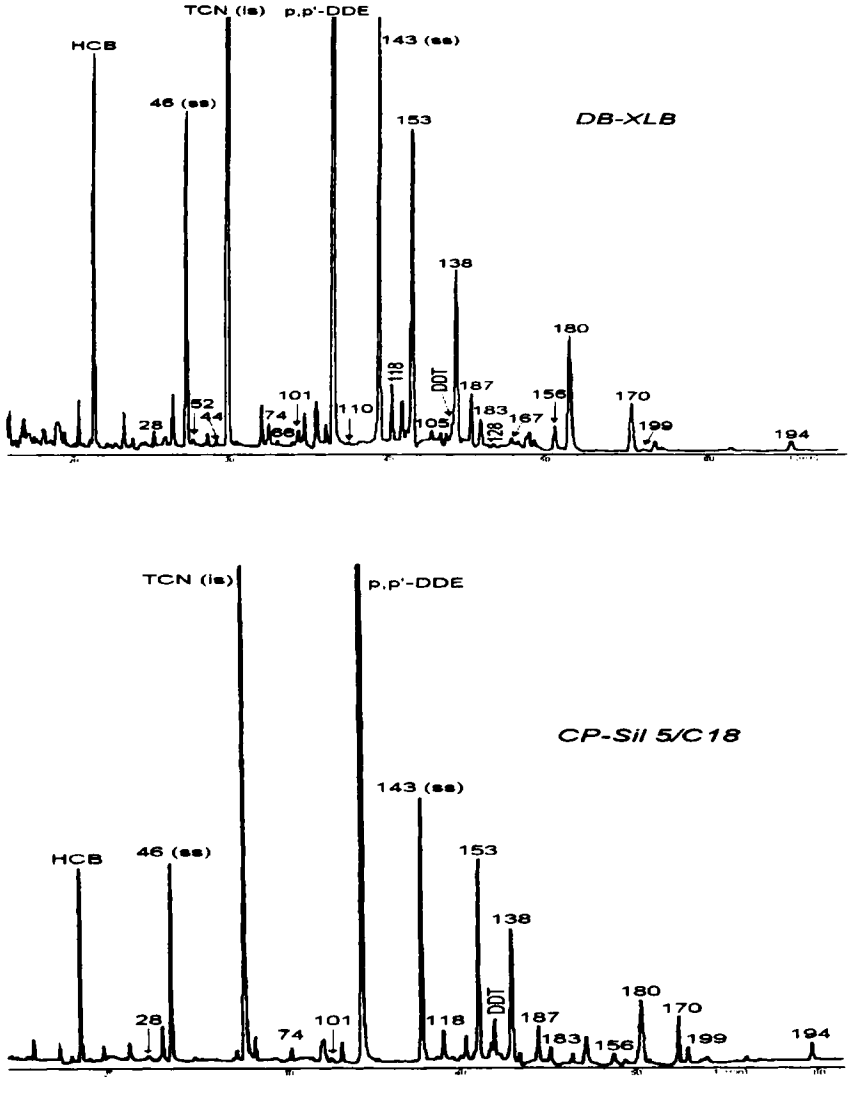

FIGURE 1 GC-ECD chromatograms of a typical human serum sample analysed on a 60 m DB-XLB column and a 50 m CP-Sil 5/C18. Individual CBs are identified by their IUPAC numbers

CB 156 and CB 157 were not baseline separated on CP-Sil 5/C18, but well separated on DB-XLB, thus the quantification of these 2 congeners was done exclusively on the last column. CB 170 and CB 190 were reported as co-eluting pair on a 5% phenyl stationary phase[24]. Both columns used in this study resolved this pair, but CB 190 co-eluted with CB 198 and CB 199. It can be concluded that the use of CB 198 as surrogate analyte – as indicated by other studies[8, 26] – is not recommended, due to possible overestimation. CB 46 and CB 143, used as surrogate standards in this study, were checked for possible co-elution with other CBs on both columns. No co-elution with CBs present in serum was found.

On a 50 m CP-Sil 5/C18 column, separation of CB 105 and CB 153 was not baseline achieved. The separation was improved on DB-XLB (with inversion of elution order), thus the concentrations reported for CB 105 were based only on results from XLB column. The elution order of CB 183 and CB 128 was different on the columns used, with better separation from possible interferences on the XLB column. Furthermore, CB 167 was found to have an interference on CP-Sil 5/C18, which was resolved on XLB. Thus the concentration reported for CB 167 was based only on the latter column.

Both columns separated CB 138 from CB 164 (when compared with a 5% phenyl stationary phase), but failed to separate it from CB 163. However, the concentration of CB 163 was reported at least 15 times lower than CB 138. Hence, the reported concentration of CB 138 was not corrected. Furthermore, p,p'-DDT was not baseline separated from CB 138 on XLB column, values of CB 138 on this column being up to 10 % higher than the values calculated from CP-Sil 5/C18. HCB was calculated from the CP-Sil 5/C18 column, due to higher values (up to 50%) from interferences on XLB column. Considering these arguments, a good reproducibility for quantification using both columns was achieved.

Analysis of individual serum samples

The method was validated on 7 individual human serum from Romania and one batch of pooled human serum from Belgium. The results are given in Table II. The concentration reported for each compound, was the average of the values obtained from the two GC columns when the difference between the values was less then 20%, otherwise, the lower level was reported. Congeners no. 18, 31, 44, 149 and 157 could not be detected in any of the samples and for CB no. 28, 52, 66, 99, 101, 105 and 110 some measurements were under the detection limit. POP concentrations were higher in samples from Romania, as they might still be in use or because of more recent restrictions (years '80), whereas they were banned in Western Europe two decades ago.

This method is currently used in a monitoring project for POPs in human serum in Flanders, Belgium and in a study on CBs concentration in maternal and umbilical cord serum.

CONCLUSION

The clean-up procedure for the analysis of selected POPs by solid-phase disk extraction provides an effective method for monitoring a large number of samples. The use of commercially available pre-packed SPE cartridges reduces significantly the time required for the sample preparation and minimises cross contamination from high-level samples and glassware. Moreover, this clean-up method requires relatively small quantities of expensive and hazardous solvents. With concentration on C_{18} cartridge, removal of interferences by sulphuric acid wash and silica gel, and quantification by HRGC/ECD, this optimised method can be used for exposure assessment.

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