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ORIGINAL ARTICLE

VALIDATION OF THE ANALYTICAL METHOD FOR THE SIMULTANEOUS DETERMINATION OF SELECTED POLYBROMINATED DIPHENYL ETHERS, POLYCHLORINATED BIPHENYLS AND ORGANOCHLORINE PESTICIDES IN HUMAN BLOOD SERUM BY GAS CHROMATOGRAPHY WITH MICRO-ELECTRON CAPTURE DETECTOR

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ABSTRACT

Background. Polybrominated diphenyl ethers (PBDEs) as other persistent organic pollutants like polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) pose a significant hazard to human health, mainly due to interference with the endocrine system and carcinogenetic effects. Humans are exposed to these substances mainly through a food of animal origin. These pollutants are globally detected in human matrices which requires to dispose reliable and simple analytical method that would enable further studies to assess the exposure of specific human populations to these compounds.
Objective. The purpose of this study was to modify and validate of the analytical procedure for the simultaneous determination of selected PBDEs, PCBs and OCPs in human blood serum samples.
Material and Methods. The analytical measurement was performed by GC-μECD following preparation of serum samples (denaturation, multiple extraction, lipid removal). Identity of the compounds was confirmed by GC-MS.
Results. The method was characterised by the appropriate linearity, good repeatability (CV below 20%). The recoveries ranged from 52.9 to 125.0% depending on compound and level of fortification. The limit of quantification was set at 0.03 ng mL^{-1} of serum.
Conclusions. The modified analytical method proved to be suitable for the simultaneous determination of selected PBDEs, PCBs and OCPs in human blood serum by GC-μECD with good precision.

Key words: *validation method, chromatography, human serum, PCBs, PBDEs, OCPs*

STRESZCZENIE

Wprowadzenie. Obecność w środowisku polibromowanych difenyloetrów (PBDE), podobnie jak innych trwałych zanieczyszczeń organicznych, do których zaliczane są polichlorowane bifenyle (PCB) i pestycydy chloroorganiczne stanowi istotne zagrożenie dla zdrowia ludzi. Substancje te mają m.in. zdolność do zaburzania równowagi układu hormonalnego i wywoływania efektów nowotworowych. Głównym źródłem narażenia ludzi na te substancje jest żywność, głównie pochodzenia zwierzęcego. Związki te wykrywane są w wielu matrycach biologicznych, co stwarza potrzebę dysponowania wiarygodną i prostą metodą analityczną, która umożliwiłaby ocenę narażenia różnych populacji na te związki.
Cel badań. Celem pracy była modyfikacja i walidacja metody analitycznej przydatnej do jednoczesnego oznaczania stężeń wybranych kongenerów PBDE i PCB, a także pestycydów chloroorganicznych w surowicy krwi u ludzi.
Materiał i metody. Oznaczanie stężeń wybranych związków prowadzono za pomocą GC-μECD po uprzednim przygotowaniu próbki surowicy (denaturacja, kilkukrotna ekstrakcja, usunięcie tłuszczu). Potwierdzanie tożsamości związków wykonywano z zastosowaniem GC-MS.
Wyniki. Metoda charakteryzuje się odpowiednią liniowością, dobrą powtarzalnością (CV poniżej 20%). Uzyskane wartości odzysków mieściły się w zakresie od 52,9 do 125% w zależności od badanej substancji i poziomu fortyfikacji. Granica oznaczalności dla wszystkich badanych związków wynosi 0,03 ng mL^{-1} surowicy.
Wnioski. Zaprezentowana metoda analityczna została uznana za odpowiednią do jednoczesnego oznaczania wybranych kongenerów PBDE, PCB oraz pestycydów chloroorganicznych w surowicy krwi metodą GC-μECD.

Słowa kluczowe: *walidacja metody, chromatografia, surowica ludzka, PCB, PBDE, pestycydy chloroorganiczne*

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INTRODUCTION

Humans are constantly exposed to the large number of ubiquitous chemicals. Persistent organic pollutants (POPs) are chemical substances, that have been identified as environmental contaminants with global distribution. Because of their lipophilicity and persistent in environment POPs bioaccumulate in fat tissue and biomagnify in food chains [22, 35]. Exposure to POPs may be associated with a wide variety of human health disorders. Many of them have been classified as endocrine disruptors (EDs) due to their interference with normal hormone function [33, 36, 37]. POPs include, among others: polychlorinated biphenyls (PCBs), hexa-, hepta- and octa- brominated diphenyl ethers, hexachlorobenzene (HCB), isomer β-hexachlorocyclohexane (β-HCH) and DDT, including its metabolites – DDD and DDE, etc.

PBDEs belong to brominated flame retardants (FRs), which since 1970s have been added to variety of commonly used products, to reduce the development of fire [5, 13, 39]. Theoretically there are 209 possible PBDEs, so called congeners, some of them are present in variety of consumer products, such as electronics (TV-sets, computers), household appliances [12], foam padding used for mattresses, furniture and carpet production, paints and plastics [26]. PBDE concentrations have decreased in Europe during the last decades [4, 28] but they are still being globally detected in food, household dust and human matrices [8, 14]. Humans are mainly exposed to these pollutants through food intake, especially food of animal origin [6]. Toxicological studies on animal models suggest that the exposure to PBDEs is associated with endocrine disruption, increased risk of reproductive risk disorders, immunotoxic effects and behavioral problems [3, 9].

Similar adverse effects on human endocrine systems have also polychlorinated biphenyls (PCBs) and their metabolites, even at low levels [10, 18], some PCBs have recently been classified by the IARC as carcinogenic to humans (Group 1) [18, 27]. Most of 209 potentially possible PCB congeners, are chemically stable and might be transferred into the food chains. PCBs have been used extensively since 1930 in a variety of application, e.g. dielectric and coolant fluids [38]. Since early 1970s use of these compounds in the USA has been prohibited. European countries in which PCBs had been produced are the UK, Germany, Italy, France, Spain and Slovakia. The use of PCBs as a raw material or chemical intermediate has been banned in the EU since 1985 (85/467/EEC, 6th amendment to Directive 76/769/EEC). The most important regulations – Council Directive 96/59/EC and HELCOM Recommendation 6/1 have been fully implemented only by the EU-Countries. Consequently, the levels of these compound in humans show decreasing tendency during the last decades [21]. However due to widespread use in the past, PCBs are still routinely detected in human tissues, breast milk, blood, in wildlife and environmental samples [14].

Organochlorine pesticides (OCPs) are another environmental pollutants with potential toxic effects on humans and wildlife. OCPs, such as DDT and its metabolites DDE and DDD, have been classified as possible human carcinogens (Group 2A) [30]. Humans are exposed to these chemicals, like others chalogenated POPs, through food chain, especially products which contain fat. Some of OCPs such as HCB, hexachlorocyclohexane (HCH) and DDT were among oldest, and most environmentally destructive pesticides used in the past [7, 19]. Most of these compounds have been prohibited for use due to their toxic effects [20], but due to its low price and good efficacy, some OCPs, such as DDT, are still used in malaria endemic countries.

A wide range of analytical methods has been developed for determination of POPs in human specimens [1, 31]. Due to similarities in physicochemical properties of these chemicals (e.g. hydrophobicity, lipophilicity, relatively good thermal stability), some steps in various 'traditional' analytical methods are almost identical [23, 34]. Generally, analytical methods include sample preparation: extraction (for example liquid-liquid extraction or solid-phase extraction) and clean-up of extracts which are followed by analysis with suitable detector. For identification and quantification most researchers used GC-MS with electron capture negative ionization (ECNI) and electron ionization (EI) or GC-ECD. The latter technique offers great sensitivity and selectivity, however confirmatory techniques are required. For determination of PBDEs and PCBs LC-MS technique can be used and it is the best analytical tool when sample volume is small [2, 32]. However for routine analysis is needed fast, simple and inexpensive method for determination of this compounds in blood samples.

The objective of this study was to optimize and validate a simple, quick and efficient method for the determination of selected POPs in human blood serum samples with adequate precision and sensitivity.

MATERIAL AND METHODS

Material

Pooled human blood serum collected as waste after the diagnostic studies was the study material. The serum was stored in the temperature of -24ºC up to analysis. This material was used to validate a method of determination of seven PBDE congeners: BDE-28 (2,4,4'-tribromodiphenyl ether), BDE-47 (2,2',4,4'-tetrabromodiphenyl ether), BDE-99 (2,2',4,4',5-pentabromodiphenyl ether), BDE-100 (2,2',4,4',6-pentabromodiphenyl

ether), BDE-153 (2,2',4,4',5,5'-hexabromodiphenyl ether), BDE-154 (2,2′,4,4′,5,6′-hexa-bromodiphenyl ether), BDE-183 (2,2′,3,4,4′,5′,6-hepta-bromodiphenyl ether), five PCB congeners: CB-77 (3,3',4,4'-tetra-chlorobiphenyl), CB-138 (2,2',3,4,4',5'-hexachlorobi-phenyl), CB-153 (2,2',4,4',5,5'-hexachlorobiphenyl), CB-170 (2,2',3,3',4,4',5-heptachlorobiphenyl), CB-180 (2,2',3,4,4',5,5'-heptachlorobiphenyl) and the follow-ing organochlorine pesticides: HCB (hexachloroben-zene), β-HCH (*beta*-hexachlorocyclohexane), γ-HCH (*gamma*-hexachlorocyclohexane), *p,p'*-DDE (dichlo-rodiphenyldichloroethylene), *p,p'*-DDT (dichlorodiphe-nyltrichloroethane), and *p,p'*-DDD (dichlorodiphenyldi-chloroethane).

Chemicals

The following reagents and solvents were used: n-hexane for organic trace analysis (Merck), tert-butyl methyl ether (MTBE) obtained from Merck, 2-propanol (POCH), KCl (Merck), concentrated sulfuric acid (Merck) and 6M hydrochloric acid (Merck). The following certified standards were used: BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 dissolved in n-nonane purchased from the Cambridge Isotope Laboratories; PCBs (CB-77, CB-153, CB-138, CB-180, CB-170) purchased from the Ultra Scientific; *p,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE, β-HCH and γ-HCH obtained from the Institute of Organic Industrial Chemistry; hexachlorobenzene purchased from Dr. Ehrenstorfer GmbH.

Standard solutions

All of certified standards were diluted in n-hexane. From the individual standard solutions the stock solutions and working solution were prepared which contained all of the analysed compounds.

Sample preparation

A modified version of the sample preparation method described by *Hovander* et al. [16] was applied. The modification included omitting of the first step of clean-up with methanol:chloroform and chloroform, as not influencing the recovery. The 2 or 3 mL of serum samples were denatured with 6M HCl (1 mL) and 2-propanole (3 or 4 mL). Then samples were extracted with n-hexane:MTBE (3 or 4 mL) and centrifuged at room temperature at 3200 *g* for 5 min. The extraction was repeated two more times. Additionally, sample was stirred and centrifuged after each extraction with hexane:MTBE. Then the organic phase was collected. The collected organic phases were evaporate to dryness. In the dry residue content of the fat was determined gravimetrically. The dry residue was dissolved in n-hexane (4 mL) and extracted with concentrated sulphuric acid (2 mL) and centrifuged for 5 min. The hexane phase was transferred to a new test tube and extracted once more. The combined extracts were concentrated and analysed by GC-μECD. Whole procedure is presented on Figure 1.

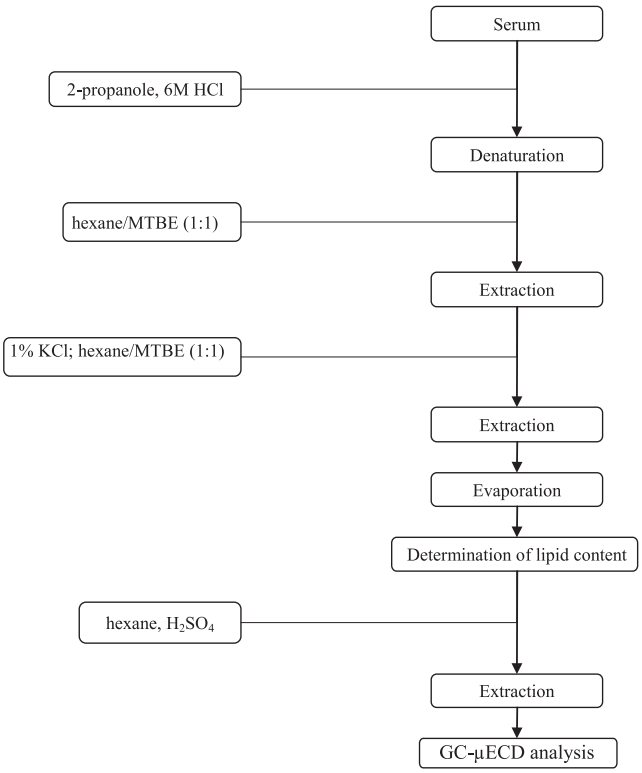

Figure 1. Diagram for preparation of blood serum sample

Chromatographic conditions

The gas chromatography (GC) analysis was performed using an Agilent Technologies 6890N GC, equipped with an Agilent Technologies 7683B autosampler, a micro-electron capture detector (μECD) and a PTV split-splitless injector operating in the splitless mode. We used a HP-5 Phenyl Methyl Siloxane (30 m x 250 μm x 0.25 μm) column with helium as a carrier gas at a constant flow rate of 3.7 mL min^{-1}, and nitrogen as a make-up gas at a flow rate of 60 mL min^{-1}. The injector temperature ramp programme in 'solvent vent' mode was: 40ºC (0.2 min) – 700ºC min^{-1} – 220ºC (1 min) – 700ºC min^{-1} – 260ºC (2 min). The GC oven temperature ramp programme mode was: 70ºC (1.7 min.) – 30ºC min^{-1} – 210ºC (0 min.) – 5ºC min^{-1} – 300ºC (5 min). The temperature of detector was set at 250ºC. The 5 μL of the sample was injected. Identification of the compounds was based on their retention times.

The confirmation of the compounds identity was conducted by gas chromatography coupled with the mass spectrometric detector (ion-trap Varian 4000) equipped with a different column (DB-5MS). The working conditions of GC-MS used for confirmation of identity of PCBs and PBDEs were as follows: injector temperature – 250ºC; carrier gas – helium; injected sample volume – 2 μL; ion trap temperature – 200ºC. The temperature ramp programme mode was: 70ºC (1 min); 30ºC min^{-1} – 170º C, 8ºC min^{-1} – 300ºC (15 min). Identification of the compounds was based on their retention times and selected characteristics ions.

The retention times of all compounds analysed by GC-μECD and GC/MS systems and their characteristic ions are shown in Table 1.

Table 1. The retention times of the individual compounds analysed using GC-μECD and corresponding retention times with selected characteristic ions examined using a gas chromatograph coupled with mass spectrometric detector (GC/MS)

Compound	GC-μECD	GC/MS	
	retention time (min)	retention time (min)	characteristic jon (m/z)
HCB	8.02	16.40	284
β-HCH	8.20	17.00	181
γ-HCH	8.29	18.50	181
p,p'-DDE	11.32	35.23	246
p,p'-DDD	12.16	35.73	235
p,p'-DDT	12.99	38.93	235
CB-77	11.50	14.06	292
CB-153	12.47	15.02	484
CB-138	13.11	15.65	360
CB-180	14.79	17.76	394
CB-170	15.59	17.75	396
BDE-28	12.05	14.60	246
BDE-47	14.88	17.32	486
BDE-100	17.39	19.03	406
BDE-99	18.12	19.78	564
BDE-154	20.32	21.09	484
BDE-153	21.40	22.24	360
BDE-183	24.73	25.35	722

Validation procedure

The following validation parameters such as linearity, accuracy (expressed as recovery for two levels of fortification), precision (repeatability), limit of quantification, and also measurement uncertainty were assessed. The present study was carried out according to validation criteria [11, 35].

RESULTS AND DISCUSSION

Linearity

The calibration curves consisted of six points for all study compounds, except *p,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE. Due to wide range of environmental levels of these compounds, ten-points calibration curves were applied. Each of concentrations was injected three times. The concentrations of the respective standard solutions used to prepare calibrations curves are shown in Table 2. The correlation coefficients met validation criteria.

Accuracy

The accuracy of the method has been reported as mean recoveries and was investigated by standard addition experiment. The samples were fortified by adding a known quantity of analytes. Recoveries were determined at two levels of fortification: 0.13 ng and 5 ng per mL of serum for all compounds except *p,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE and , 5 ng and 125 ng per mL of serum for *p,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE. The Dixon test [17, 25] was using for the elimination of the outliers. Mean recoveries of fortification level at 0.13 ng mL^{-1} ranged from 57.5 (BDE-28) to 102% (CB-77), as shown in Table 3. Similar tendencies can be observed in case of low recovery for BDE-28 and BDE-47, observed by other researchers, for example *Hovander* et al. [16] and *Loconto* et al. [29], that lower substituted PBDE congeners showed lower recovery. In the case of the PCBs similar relationship was observed by *Keller* et al. for PCB that recoveries decreased with increasing chlorination of PCBs (except of CB-170 and CB-180) [24].

GC-μECD chromatograms of the mixture containing all tested compounds and fortified blood serum sample extract are shown in Figure 2 and Figure 3.

Eight samples were fortified at level of 50 ng mL^{-1}. The mean recoveries ranged from 52.9 (BDE-183) to 125% (*p,p'*-DDE), as shown in Table 4.

Repeatability

The repeatability of the method was calculated based on the standard deviation ($SD_{repeat.}$) of series of measurements made by the same analyst, in a short period of time. The results are shown in Tables 4 and 5. Coefficients of variation (CV) were below 20% in any case. The method was characterized by good precision according to the modified *Horwitz* equation [15].

Limit of quantification

The limit of quantification (LOQ) has been assumed as the lowest calibration point and was approximately 0.03 ng mL^{-1} of serum.

Table 2. Calibrations points for particular compounds [ng mL^{-1}]

Compound	1	2	3	4	5	6	7	8	9	10
p,p'-DDT	0.095	0.24	0.98	1.97	3.94	12.31	27.36	54.72	79.80	114.0
p,p'-DDE	0.095	0.24	0.98	1.97	3.94	12.31	24.62	49.25	71.82	102.6
p,p'-DDD	0.093	0.23	0.97	1.95	3.89	12.17	24.34	48.67	70.98	101.4
β-HCH	0.100	0.25	1.04	2.09	4.18	13.06	-	-	-	-
γ-HCH	0.102	0.25	1.06	2.12	4.25	13.27	-	-	-	-
HCB	0.092	0.23	0.96	1.92	3.83	11.98	-	-	-	-
CB-77	0.096	0.24	1.00	1.99	3.98	12.45	-	-	-	-
CB-138	0.094	0.23	0.97	1.95	3.90	12.18	-	-	-	-
CB-153	0.095	0.24	0.99	1.98	3.96	12.36	-	-	-	-
CB-170	0.092	0.23	0.96	1.92	3.85	12.02	-	-	-	-
CB-180	0.093	0.23	0.97	1.94	3.89	12.14	-	-	-	-
BDE-28	0.094	0.24	0.98	1.96	3.92	12.24	-	-	-	-
BDE-47	0.094	0.24	0.98	1.96	3.92	12.24	-	-	-	-
BDE-99	0.094	0.24	0.98	1.96	3.92	12.24	-	-	-	-
BDE-100	0.094	0.24	0.98	1.96	3.92	12.24	-	-	-	-
BDE-153	0.094	0.24	0.98	1.96	3.92	12.24	-	-	-	-
BDE-154	0.093	0.23	0.97	1.94	3.89	12.14	-	-	-	-
BDE-183	0.092	0.23	0.96	1.92	3.84	12.00	-	-	-	-

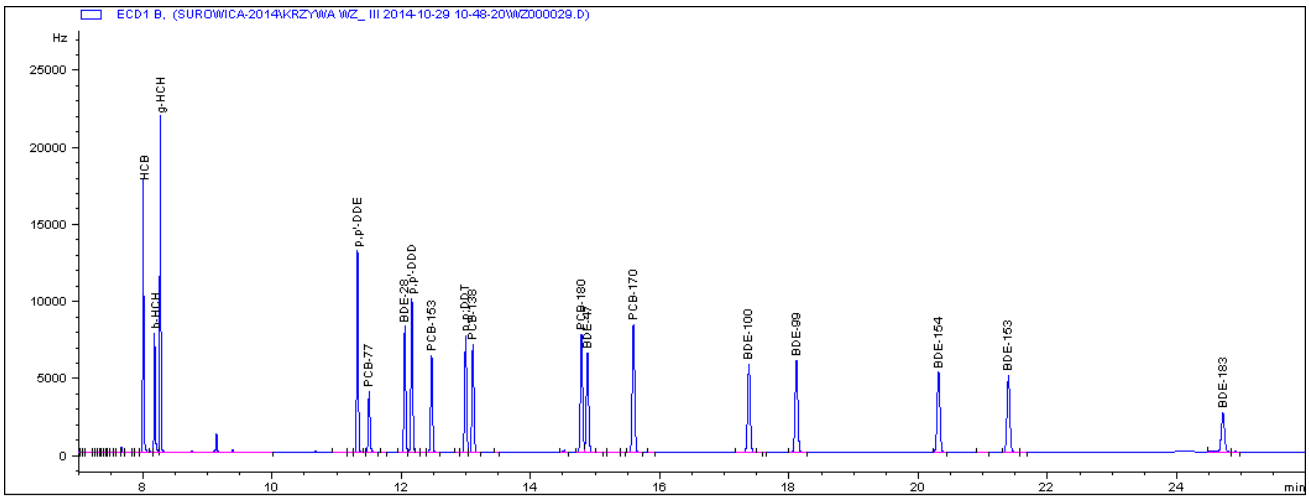

Figure 2. GC-µECD chromatogram of the standard mixture containing all determined compounds in approx. 12 ng mL^{-1}

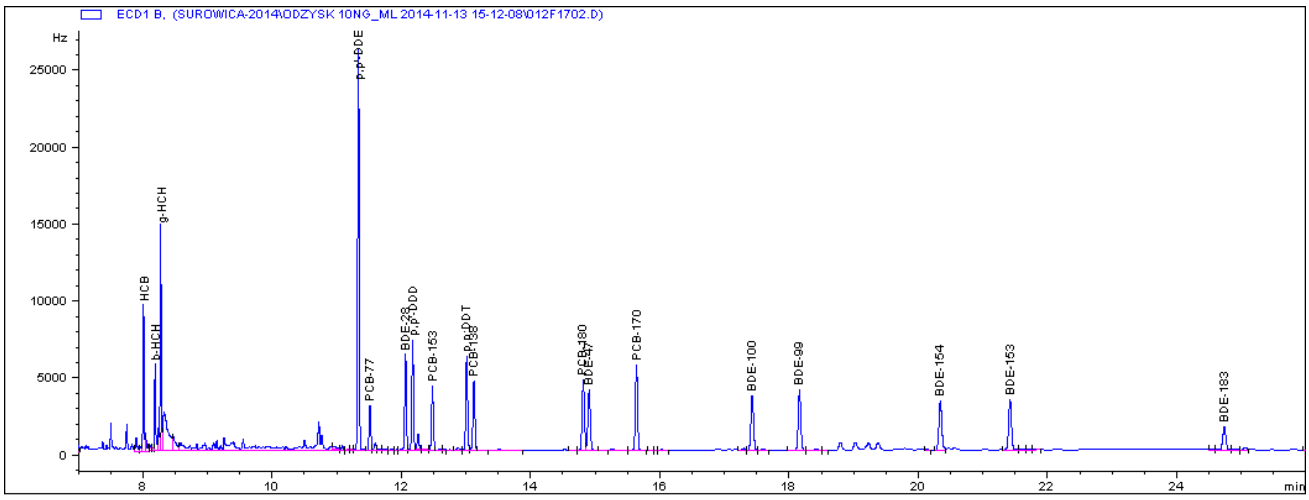

Figure 3. GC-µECD chromatogram obtained for analysis of the fortified blood serum sample of 18 compounds approx. 10 ng mL^{-1}

Table 3. Summary of validation parameters; fortification – 0.13 ng mL^{-1} in serum

Compound	Mean recovery [%]	n	SD [ng mL^{-1}]	CV [%]	U [%]
HCB	82.5	9	0.05	16.5	31
β-HCH	78.6	9	0.02	5.2	12
γ-HCH	61.9	7	0.05	20.8	50
CB-77	102.3	9	0.11	26.1	41
CB-138	83.8	9	0.04	13.0	24
CB-153	83.2	9	0.02	5.6	20
CB-170	79.5	9	0.04	8.5	18
CB-180	74.9	9	0.03	13.2	27
BDE-28	57.5	7	0.01	4.4	12
BDE-47	60.7	9	0.02	9.4	23
BDE-99	87.7	9	0.05	14.0	26
BDE-100	94.3	9	0.04	11.2	16
BDE-153	67.2	9	0.03	12.3	27
BDE-154	91.2	9	0.02	6.6	11
BDE-183	62.9	9	0.02	8.7	22

Table 4. Summary of validation parameters; fortification – 5 ng mL^{-1} in serum

Compound	Mean recovery [%]	n	SD [ng mL^{-1}]	CV [%]	U [%]
HCB	76.2	8	1.3	16.8	35
β-HCH	88.1	8	0.6	6.1	18
γ-HCH	77.7	8	1.2	14.5	29
p,p'-DDE	125.0	8	1.7	13.3	17
p,p'-DDD	96.5	8	0.5	4.7	8
p,p'-DDT	78.3	8	0.4	5.3	11
CB-77	83.5	8	0.3	3.8	7
CB-138	80.3	8	0.5	6.5	13
CB-153	80.0	8	0.2	2.4	4
CB-170	84.8	8	0.2	2.9	6
CB-180	85.5	8	0.3	3.5	7
BDE-28	92.5	8	0.4	4.0	7
BDE-47	85.3	8	0.3	3.3	7
BDE-99	91.1	8	0.3	2.8	6
BDE-100	84.1	8	0.3	3.5	7
BDE-153	88.6	8	0.4	4.0	8
BDE-154	96.4	8	0.4	3.9	6
BDE-183	52.9	8	0.3	6.1	18

For recovery testing at level of 125 ng mL^{-1} the total number of samples fortified was 16. The mean recoveries for *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, were respectively 87.1, 95.1 and 94.9% (Table 5).

Table 5. Summary of validation parameters; fortification – 125 ng mL^{-1} in serum

Compound	Mean recovery [%]	n	SD [ng mL^{-1}]	CV [%]	U [%]
p,p'-DDE	87.1	16	4.9	5.6	20
p,p'-DDD	95.1	16	3.5	3.7	12
p,p'-DDT	94.9	16	6.2	6.6	20

Measurement uncertainty

The relative expanded uncertainty was calculated according to the equation: $U = k \times u_c$ (k=2) in the 95% confidence interval, assuming squared probability of results distribution, where u_c - combined standard uncertainty of series of repeated measurement. The combined standard uncertainty (u_c) was calculated according to the formula on the concentration of the test compound, taking into account recovery. For each parameter of the formula standard uncertainty (u) was calculated according to the appropriate distribution (square or triangular) [11]. The estimated uncertainty values for tested compounds for different levels of fortification are presented in Tables 3, 4 and 5, were in the ranged from 6% to 50%.

CONCLUSIONS

The result of validation of presented analytical method prove its usefulness for biomonitoring purposes. The omission of the blood serum clean-up step had no adverse effect on the recovery values. On the basis of the determined validation parameters it can be concluded that the method is characterized by good repeatability and can be successfully applied for the simultaneous determination of eighteen organohalogen compounds (i.e. PBDEs, PCBs, OCPs) in blood serum samples, allowing to estimate the human exposure to these substances. Presented analytical method is relatively quick and simple.

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Conflict of interest

The authors declare no conflict of interest.

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