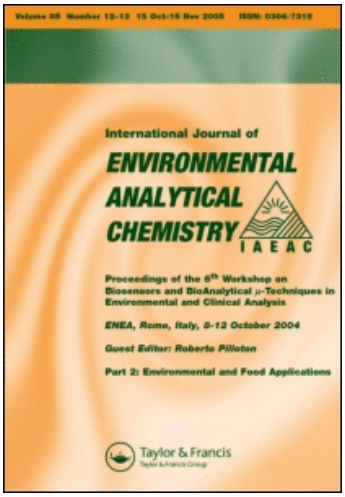

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Simultaneous Separation and Determination of Polychlorinated Biphenyl Congeners and other Chlorinated Hydrocarbon Residues in Human Matrices using Gel Permeation or Adsorption Chromatographic Clean-Up and GC-MS Quantification

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SIMULTANEOUS SEPARATION AND DETERMINATION OF POLYCHLORINATED BIPHENYL CONGENERS AND OTHER CHLORINATED HYDROCARBON RESIDUES IN HUMAN MATRICES USING GEL PERMEATION OR ADSORPTION CHROMATOGRAPHIC CLEAN-UP AND GC-MS QUANTIFICATION

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Two methods are described for the analysis of organochlorine compounds (OCs) in human matrices (adipose tissue and serum). These comprise the extraction, clean-up and quantification by gas chromatography-mass spectrometry in Selected Ion Monitoring-mode. The procedures involve liquid-liquid extraction for serum and column percolation for adipose tissue. The serum clean-up involves adsorption chromatography through deactivated silica gel. The solvent system, consisting of 30 mL *n*-hexane, followed by 10 mL *n*-hexane:dichloromethane (80:20, v/v) and 10 mL *n*-hexane:dichloromethane (40:60, v/v), gives acceptable recoveries, but is not suitable for the extraction of dieldrin and lindane (γ-HCH). An optimisation of gel permeation chromatography (GPC) is presented for separation of OCs from extractable adipose tissue lipids. The high efficiency of the GPC provides very clean extracts for GC-MS analysis and yields high recoveries for the individual compounds studied (mean recovery 89 % for pesticides and polychlorinated biphenyls).

Keywords: Adipose tissue; gas chromatography; gel permeation chromatography; mass spectrometry; organochlorine pesticides; polychlorinated biphenyls; serum

INTRODUCTION

Organochlorine pesticides and polychlorinated biphenyls (PCBs) are two classes of organochlorine compounds (OCs) that are structurally and toxicologically

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related. With the increasing number of pollutants and the possibility of interaction between compounds in their toxic activity, there is a need for multi-residue methods for simultaneous quantitative determination of a large number of chlorinated hydrocarbons. For this study 10 PCB congeners and 10 organochlorine pesticides are selected based on their occurrence, persistence, toxicity and their prevalence in human matrices [1–3]. Two methods are described for the analysis of the pollutants under investigation in human matrices (adipose tissue and serum). Different procedures are used for different matrices depending on the lipid load. These comprise the extraction, clean-up and quantification by gas chromatography-mass spectrometry (GC-MS) in Selected Ion Monitoring (SIM)-mode. The method involves liquid-liquid extraction for serum and column percolation for adipose tissue. The serum clean-up involves adsorption chromatography through deactivated silica gel. An optimisation of gel permeation chromatography (GPC) is presented for separation of OCs from extractable adipose tissue lipids.

In contrast to other chromatographic techniques, GPC is unique in that separations are based primarily on differences in molecular size. The molecular weight of most pesticides varies between 200 and 400 Dalton, while that of most lipids is between 600 and 1500 Dalton. In addition, most pesticides with high molecular weights contain a high content of chlorine and are more compact than lipids. Thus, GPC should effectively separate lipids from most OCs [4–6]. A GPC clean-up technique for pesticide residue determination was introduced by Stalling *et al.* in 1972 [4]. The applicability of GPC in the area of trace analysis of organic contaminants has been published before [3,7–9]. It is a clean-up technique recommended by the Association of Official Analytical Chemistry for organochlorine pesticides in animal fat [10]. We have modified parameters as packing material, elution solvent system and flow rate to better accomodate the multi-residue analysis of chlorinated hydrocarbons in human adipose tissue. The most frequently cited stationary phase is the polystyrenedivinylbenzene copolymer Bio Beads S-X3 [11–13].

MATERIALS AND METHODS

Apparatus and reagents

GPC was performed on a linear solvent delivery system including a Pharmacia-LKB Pump P-500, low-pressure 1/16 tube couplings, flanged PTFE fittings for 1/16 in. tubing and 1/16 in. od × 0.01 mm id PTFE tubing (Pharmacia Bio-

tech, Roosendaal, the Netherlands). The connection was made using a four-way introduction rotary valve (SRV-4, Pharmacia Biotech, Roosendaal, the Netherlands) and a Rheodyne 7125 injector with a 5 mL loop (Alltech, Laarne, Belgium). A glass column (50 cm × 25 mm id) slurry packed with 60 g of 200–400 mesh Bio Beads S-X3 (Bio-Rad Laboratories, Richmond, CA) was compressed to a bed length of 37 cm. Elution was performed with *n*-hexane:dichloromethane (1:1, v/v) at 1.5 mL/min. Fractions were collected in a LKB 2212–010 Helirac fraction collector (LKB Produkter, Bromma, Sweden).

Adsorption chromatography was performed on 70–230 mesh silica gel (Merck, Darmstadt, FRG) in a glass 50 mL column (18 cm × 9 mm id, with stopcock) using sodium sulfate (Merck, Darmstadt, FRG) and glass wool (dimethyldichlorosilane-treated).

We used a Hewlett Packard 5890 a series II Plus gas chromatograph (HP, Palo Alto, USA) equipped with a HP 5972A quadrupole mass spectrometric detector, autosampler and a fused silica DB-XLB capillary column (J&W Scientific, Folsom, USA) of 60 m × 0.25 mm id × 0.2 μm film thickness. The GC temperature programme was as follows: initial 80 °C held for 2 min, then to 220 °C at a rate of 30 °C min^{-1}, 3.5 °C min^{-1} to 250 °C, kept for 2 min and then 1.5 °C min^{-1} to 270 °C, held for 6 min. The pressure pulse programme consisted of an initial pressure of 14.8 psi held for 0.1 min, 99 psi min^{-1} to 14.8 psi, kept for 0.5 min and then 0.48 psi min^{-1} to 28.1 psi, kept for 6 min. Carrier gas was helium (N56 grade, Air Liquide, Liege, Belgium) at a linear velocity of 22.3 cm sec^{-1}. Two μL was injected splitless at injector temperature of 280 °C.

Acetone, benzene, dichloromethane, diethyl ether, *n*-hexane, methanol were purchased pesticide grade (Merck, Darmstadt, FRG). All glasware used was presoaked in sulphochromic acid for at least 24 hours. Prior to use, the treated glassware was rinsed with acetone and the solvent with which it would subsequently be in contact.

Reference standards included all compounds under investigation and were purchased in crystalline form from J.T.Baker (Deventer, the Netherlands). Chrysene-d_{12} was obtained from Cambridge Isotope Labs (Woburn, MA, USA) and was used as an internal standard. Individual stock solutions of 1 μg/μL were prepared in methanol.

Sample preparation

Sampling was conducted in collaboration with the University Hospital of Antwerp (UZA, Edegem, Belgium). Human subcutanous adipose tissue was collected during autopsy. Different batches of human blood serum were obtained from the Blood Transfusion Center. Blood was collected in a vacuum system

tube, transported in a cooling-pail, and centrifuged within 24 hours during 15 minutes at 3000 rpm. All samples were kept frozen at –20 °C until analysed. The samples were thawed and weighed. Average weights were roughly an aliquot of 10 g of the pooled serum and 1 g of adipose tissue. Samples were spiked with an organochlorine compound standard solution and chrysene-d_{12} as internal standard. After spiking, the concentration of each product was 20 ng/g adipose tissue and 2 ng/g serum. Before extraction the spiked samples were equilibrated by ultrasonic treatment for 20 min.

Serum analysis

Deactivation of silica gel

Twenty g active silica gel was weighed into a 250 mL beaker. The beaker was covered with aluminum foil into which several holes in the foil were punched, and left at 130 °C for 24 hours. the silica gel was cooled in a vacuum desiccator. Dried silica gel was weighed into a flask with a Teflon-lined screw cap. Water was added to constitute 3% of total weight (e.g. 0.62 g H_2O added to 20 g silica gel). The Teflon-lined cap was wrapped with tape to seal the flask. The wetted gel was shaked until no longer clumps were evident. The flask was gently rotated for 3 hours on a mechanical rotavapor and left tightly capped overnight before use. Silica gel prepared in this manner maintains its elution characteristics during 7 days stored on bench top.

Extraction of serum

Two extraction methods were evaluated. For both methods, 10 mL of the pooled serum was pipetted into a clean 16 × 125 mm culture tube which had a Teflon-lined screw cap and the sample was spiked (see sample preparation). Method 1 is one of the classical methods commonly used for biological samples [1]. For this method 5 mL methanol was added, the tube was capped and swirled briefly in a vortex mixer for 1 min. After addition of 10 mL *n*-hexane:diethyl ether (1:1, v/v) to the tube, the latter was capped, briefly vortexed for 1 min, and put in an ultrasone bath for 15 min. The mixture was finally centrifuged for 10 min at 3000 rpm. For the second method (based on Mes *et al.* [14]), 10 mL benzene was added prior to centrifugation for 15 min at 3000 rpm. For both methods, disposable glass Pasteur pipes were used to remove supernatant. The extraction was repeated twice. The extracts were combined and the solvent volume reduced in a vacuum oven at 30 °C. The lipid content was determined gravimetrically; 0.63 % extractable lipids for the first method (n = 8, range 0.50–0.85 %) and 0.18 % for the second method (n = 8, range 0.13–0.22 %). The

extract was then ready for further clean-up. The extraction recovery information for both methods is summarised in Table I.

TABLE I Percentage recoveries (n=4) of liquid-liquid extraction* of 20 OCs (fortification level: 2 ng/g serum)

Compound	*Method 1 (%) Recovery (RSD)*	*Method 2 (%) Recovery (RSD)*
Organochlorine Pesticides		
β-HCH	115 (6)	110 (6)
γ-HCH	interf	119 (9)
o,p'-DDT	79 (4)	71 (5)
p,p'-DDT	87 (5)	63 (5)
o,p'-DDD	87 (6)	107 (4)
p,p'-DDD	94 (5)	109 (7)
o,p'-DDE	85 (7)	87 (7)
p,p'-DDE	89 (7)	98 (6)
heptachlorepoxide	111 (9)	113 (7)
dieldrin	108 (7)	99 (8)
Polychlorinated Biphenyls		
PCB-28	113 (9)	110 (10)
PCB-52	112 (8)	112 (8)
PCB-77	76 (4)	97 (6)
PCB-101	80 (6)	81 (4)
PCB-118	74 (5)	80 (6)
PCB-126	70 (7)	72 (4)
PCB-138	69 (5)	79 (4)
PCB-153	71 (6)	76 (5)
PCB-169	63 (7)	71 (5)
PCB-180	63 (8)	70 (6)

* First step in serum analysis.

Adsorption Chromatographic clean-up

The clean-up for serum is adapted from Burse *et al.* [15] employing adsorption chromatography in deactivated silica gel. The column was prepared as follows: a column was packed with a small plug of silanised glass wool. *(1)* Anhydrous Na_2SO_4 was added to a height of 10 mm, followed by *(2)* 5.0 g of 3% deactivated silica gel, and *(3)* anhydrous Na_2SO_4 a to height of 10 mm. The column was pre-washed with 25 mL of *n*-hexane:dichloromethane (1:1, v/v) and 25 mL of hexane. Concentrated extract dissolved in 0.5 mL hexane was added. The sample was rinsed with three 0.5 mL portions of hexane. Four different solvent

systems were evaluated as elution agents. In all cases, the collected fractions were evaporated to a small volume. Chrysene-d_{12} was added as internal standard prior to GC-MS analysis. The final volume of the extract was a compromise to maximise the enrichment factor without significant evaporation loss. Recoveries of the different solvent mixtures are shown in Table II. The flow chart in Figure 1 summarises the extraction, clean-up and quantification procedure for OCs from serum.

TABLE II Percentage recoveries (n=4) of mixed OC standard solution for clean-up of human serum through silica gel (1 ng/μL, 20 μL through extraction system)

Compound	*Eluting system* [a]			
	I	*II*	*III*	*IV*
Organochlorine Pesticides				
β-HCH	26	28	15	84
γ-HCH	27	46	22	36
heptachlorepoxide	43	68	41	93
dieldrin	-	-	-	-
o,p′-DDT	101	59	43	107
p,p′-DDT	85	50	52	107
o,p′-DDE	102	84	40	111
p,p′-DDE	106	88	46	104
o,p′-DDD	13	92	47	108
p,p′-DDD	17	92	44	103
Polychlorinated Biphenyls				
PCB-28	42	66	39	109
PCB-52	55	75	40	115
PCB-77	103	84	42	108
PCB-101	105	86	37	115
PCB-118	103	86	41	104
PCB-126	102	86	43	95
PCB-138	105	83	41	100
PCB-153	104	84	44	99
PCB-169	107	81	40	103
PCB-180	113	81	43	100

[a] **I** 30 mL hexane
II 30 mL hexane:dichloromethane (80:20, v/v)
III 10 mL hexane
10 mL hexane:dichloromethane (80:20, v/v)
10 mL hexane:dichloromethane (50:50, v/v)
IV 30 mL hexan
10 mL hexane:diethane (80:20, v/v)
10 mL hexane:dichloromethane (40:60, v/v)

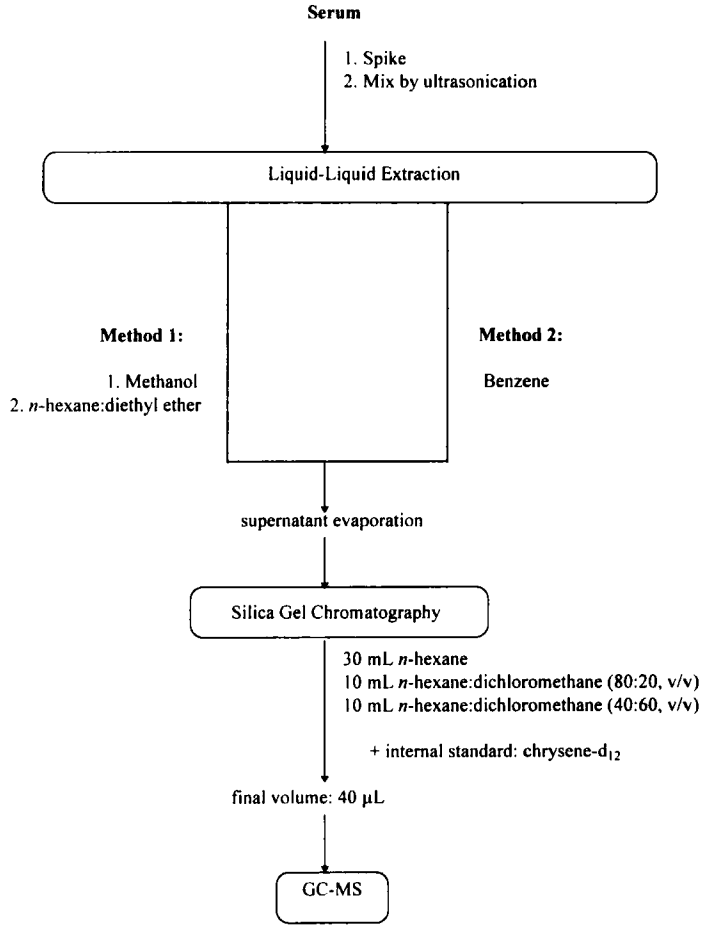

FIGURE 1 Flow chart of sample preparation for analysis of OCs in human serum

Adipose tissue analysis

Extraction

After spiking (see sample preparation) the tissue was homogenised in a mortar with a 10 times higher weight of Na_2SO_4. The mixture was transfered to an extraction column of 15 mm internal diameter and 250 mm height and eluted subsequently with 50 mL *n*-hexane:dichloromethane (1:1, v/v). After evaporation and drying of the eluate by means of a rotavapor ≤ at 30 °C, the lipid content was determined gravimetrically. The eluate was then ready for further clean-up.

Gel permeation calibration

To determine the elution profile for adipose tissue, 1 g sample was prepared as described above, diluted with *n*-hexane:dichloromethane (1:1, v/v) to 3 mL and mixed thoroughly. Then the extract was injected on the GPC system. Twenty fractions of 10 mL were collected over the 0 – 200 mL elution range in tared vials. Fractions were evaporated and weighed. Results of the human adipose tissue elution profile are shown in Figure 2. To determine OC elution profiles, a mixed OC standard solution was injected on the GPC and collected in 10 mL increments from 100 to 250 mL. After drying, chrysene-d_{12} internal standard was added, dissolved in 40 µL methanol and 2 mL was injected in the GC-MS for the final analysis. Clean-up and total extraction recoveries of the analytes are summarised in Table III.

TABLE III Percentage recoveries (n=4) of 20 compounds from fortified fat matrix (fortification level: 20 ng/g adipose tissue)

Compound	*Column Percolation (%)*	*Gel Permeation Chromatography 150–250 mL (%)*
Organochlorine Pesticides		
β-HCH	101	77
γ-HCH	98	73
o,p'-DDT	96	95
p,p'-DDT	98	77
o,p'-DDD	97	99
p,p'-DDD	96	95
o,p'-DDE	101	95
p,p'-DDE	99	87
heptachlorepoxide	99	96
dieldrin	96	96
Polychlorinated Biphenyls		
PCB-28	106	61
PCB-52	107	72
PCB-77	97	91
PCB-101	101	89
PCB-118	100	89
PCB-126	98	94
PCB-138	101	96
PCB-153	100	103
PCB-169	98	98
PCB-180	97	101

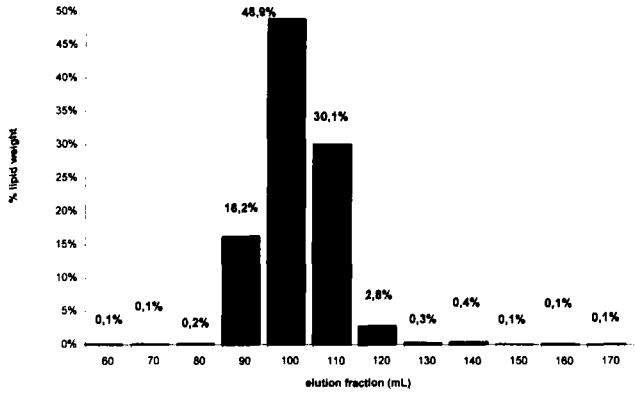

FIGURE 2 Elution pattern for 1 g extracted adipose tissue on GPC with 60 g Bio-Beads S-X3, elution solvent *n*-hexane:dichloromethane (1:1, v/v)

Sample preparation

Blanc samples and mixed standard elution profiles were employed to determine appropriate dump-and-collect fractions for OCs in human matrices. Samples were prepared as described before (see sample preparation and gel permeation calibration). The GPC column was loaded with 3 mL solution and the eluate was collected from 150 to 250 mL in a 100 mL boiling flask. This was rotary-evaporated to just dryness at ≤ 30 °C. Chrysene-d_{12} internal standard was added prior to analysis by GC-MS. A sample preparation scheme for OC analysis in adipose tissue is presented in Figure 3.

Relative response factors and calculations

A daily performance/calibration standard was used to check instrument performance, reproducibility and sensitivity. The same standard was also used to generate RFF for quantification. Two μL aliquots of an organochlorine compound standard solution were injected to obtain OC and chrysene-d_{12} (internal standard) areas. The RFF was calculated as follows:

$$RFF = (X'/X) \times (C/C')$$

Where X and X' are the area of OC and chrysene-d_{12} peaks respectively, and C and C' are the concentrations of OC and chrysene-d_{12}. After RFFs, mean standard deviation and relative standard deviation for each OC peak were calculated. Five injections of the standard mix solution before initiating an analytical run and every fifth injection designed as a standard during an analytical run are gen-

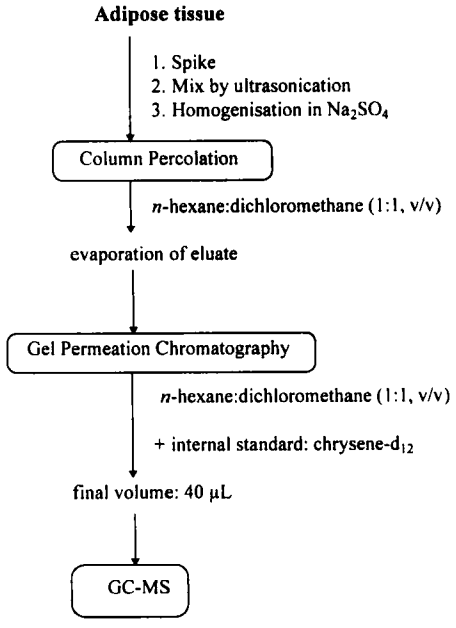

FIGURE 3 Flow chart of sample preparation for analysis of OCs in human adipose tissue

erally considered adequate for monitoring RFF values. RSD ≤ 10 % was attainable for all OC peaks. Once RFF values were generated, the concentration of any OC peak could be calculated.

GC-MS determination

The MS-instrument was operated in the electron impact (El) ionisation mode at 70 eV using SIM. A SIM-table was set up for GC-MS identification, based on the elution order of all compounds analysed. Retention times, masses and the ratio of confirmation ion intensity to quantitation ion intensity in comparison with the expected ratio for each level of chlorination were used as identification criteria. Compounds were monitored using SIM in groups determined by the number of chlorine atoms in the molecule. 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 had to be present. Ions *(m/z)* selected in the GC-MS determination of OCs and corresponding retention times are shown in Table IV. A deviation of the isotope ratio of less than ± 20 % from the theoretical value was still considered

acceptable, but usually the deviation remained within ± 10 % as described by a recent EC directive [17].

TABLE IV 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		219	181, 217	15.31
γ-HCH		219	181, 217	16.00
heptachlorepoxide		253	135, 183	20.27
dieldrin		263	108, 277	23.19
o,p'-DDT		235	165, 237	24.62
p,p'-DDT		235	165, 237	27.20
o,p'-DDD		235	165, 237	22.94
p,p'-DDD		235	165, 237	25.31
o,p'-DDE		318	176, 246	20.69
p,p'-DDE		318	176, 246	22.42
Polychlorinated Biphenyls				
Chlorine substitution pattern [a]	**Congener IUPAC N° [b]**			
2,4,4'	PCB-28 *	256	186, 258	16.77
2,2',5,5'	PCB-52 *	292	220, 290	17.61
3,3',4,4'	PCB-77 **	292	220, 292	23.74
2,2',4,5,5'	PCB-101 *	326	256, 324	21.09
2,3',4,4',5	PCB-118 *	326	256, 324	24.73
3,3',4,4',5	PCB-126 **	326	256, 324	28.81
2,2',3,4,4',5	PCB-138 *	360	290, 358	25.53
2,2',4,4',5,5'	PCB-153 *	360	290, 358	27.29
3,3',4,4',5,5'	PCB-169 **	360	290, 358	34.79
2,2',3,4,4',5,5'	PCB-180 *	394	324, 392	31.75
Internal Standard				
Chrysene-d_{12}		240	125, 241	32.48

[a] Numbering indicates level and position of chlorine substitution.
[b] Numbering system according to Ballschmiter and Zell [16]
* original "seven" PCBs selected for certification and are the basis of most environmental monitoring programmes.
** planar PCB congeners.

RESULTS AND DISCUSSION

Gas chromatography – mass spectrometry

For the determination of these analytes, a GC-MS technique was adopted to obtain a high-confidence identification of target analytes in the complex mixture. The first objective of the present study was the development of a gas chromatographic method with a single DB-XLB column that resolved as completely as possible the set of 20 OCs. The whole separation and GC run was being achieved in 36 min with the MS allowing an unambiguous identification of the different analytes. The methods described here yield good recoveries for high fortification levels of compounds analysed. At lower fortification levels (especially in case of minor planar PCBs) quantification based on $^{13}C_{12}$-labeled internal standards can give solution to avoid interfering more abundant congeners.

Extraction and clean-up efficiency

The residue analysis of OCs in complex biological matrices needs extensive analyte purification and concentration. The analysis by highly sensitive and specific GC-MS is only practicable with very clean and almost matrix-free extracts. Furthermore, a thorough clean-up minimises matrix effects on column behaviour and detector response, permits more consistent and reproducible injections and extends the lifetime of the capillary columns. Efficiency of sample clean-up, measured in terms of lipid carry-over and "cleanliness" of gas chromatograms is excellent for all evaluated methods.

Adipose tissue clean-up by gel permeation chromatography

The gel-chromatographic elution behaviour of various analytes depends on many parameters, e.g. elution strength of the solvent mixtures, the degree of soaking of the filling height of the gel bed, the elution flow rate, sample fat content etc. Minor variations lead to different results [11]. Figure 2 shows the result of the lipid elution pattern on the experimental conditions. The ordinate shows the collected volume and the abscissa the percentage of the total lipids collected. The lipids begin eluting after 90 mL eluant. After 150 mL 99.47 % of the lipid has been removed. The remainder of the lipid eluted thereafter, does not interfere with the GC quantification of the OCs. Fractionation of the chromatographic effluent shows that they elute in 150 to 250 mL. The analytes can be collected, concentrated, and injected into a GC-MS, without further additional clean-up.

From these experiments the following gelchromatographical parameters are obtained: a dump-volume of 150 mL and a collected volume of 100 mL. The narrow elution bands of lipids and OCs coupled with the ease of evaporating the eluting solvent makes this system efficient because saving time and solvents. The collection of the analytes under investigation in only one fraction results in a considerable saving of analysis time (only one GC run). Under the given conditions, GPC yields high recoveries for the individual compounds studied (mean recovery of 89% for both pesticides and PCBs). The recovery rates may be improved by further optimisation of critical steps of the clean-up.

Serum extraction by liquid-liquid chromatography

Both methods yield acceptable extraction recoveries. Denaturation with methanol and extraction with *n*-hexane:dichloromethane appeared to be more time consuming, requiring more solvent. The use of a highly toxic solvent such as benzene as employed in the alternative method, should preferably be avoided.

Serum clean-up through deactivated silica gel in adsorption chromatographic column

For the serum clean-up through silica gel, different solvent mixtures and solvent mixture gradients were evaluated. The solvent system, consisting of 30 mL *n*-hexane, followed by 10 mL *n*-hexane:dichloromethane (80:20, v/v) and 10 mL *n*-hexane:dichloromethane (40:60, v/v), gave the best OC recoveries. Only dieldrin and lindane (γ-HCH) were not obtained quantitatively. Therefore, the use of this clean-up procedure for the determination of the latter analytes is questionable. The other compounds under investigation gave acceptable recoveries (mean recovery of 102.1% and 104.8% respectively for pesticides and PCBs), leaving the serum lipids completely on the silica adsorption column. No interfering peaks were present with the GC-MS quantification.

CONCLUSION

Two methods for the analysis of organochlorine compounds (OCs) in human matrices (adipose tissue and serum) are presented. An optimisation of GPC is described for the separation of OCs from adipose tissue. Optimal GPC conditions for efficient clean-up were obtained using Bio Beads S-X3 and *n*-hexane:dichloromethane (1:1, v/v) as elution agent. The analytes of interest could be

collected and quantified by GC-MS without further clean-up. For the analysis of OCs in human serum, two extraction methods were compared. Both of them gave acceptable recoveries, but because the use of highly toxic benzene in the second method, we would prefere the slightly more laborious serum denaturation with methanol followed by liquid-liquid extraction with *n*-hexane:diethyl ether (1:1, v/v). For the serum clean-up through silica gel, different solvent mixtures and solvent mixture gradients were evaluated. The solvent system consisting of 30 mL *n*-hexane, followed by 10 mL *n*-hexane :dichloromethane (80:20, v/v) and 10 mL *n*-hexane:dichloromethane (40:60, v/v) gave the best OC recoveries, without serum lipid interferences for GC-MS quantification.

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