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Extraction and Cleanup Methods for Analysis of Phenolic and Neutral Organohalogens in Plasma

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Abstract

A method for the analysis of potential endocrine-disrupting compounds, such as phenolic halogenated compounds (e.g., chlorinated and brominated phenols) and hydroxylated PCBs, in blood plasma is presented. Neutral halogenated compounds, specifically brominated diphenyl ethers and PCBs, are also included in the evaluation. An efficient denaturation and extraction step is described, and three methods for lipid removal are evaluated. The latter includes a nondestructive method based on high-resolution gel permeation chromatography (HR-GPC), a newly developed silica gel/sulfuric acid column, and lipid removal by sulfuric acid treatment. Recoveries, based on gas chromatography with an electron capture detector (GC–ECD), were between 70 and 90% for most of the studied compounds. The recoveries of phenolic compounds were generally slightly lower than those of the neutral compounds. The sulfuric acid treatment and silica gel/sulfuric acid column gave the highest yields for acid stable compounds, although a few target compounds were lost during that treatment and all compounds were recovered with the HR-GPC method.

Introduction

The exposure of the human population to environmental organohalogen substances (OHS) has been of global concern for several decades, resulting in several reports on potential hazards and risk assessment being published (1–5). More recently, focus has been directed towards potential endocrine-disrupting compounds. Many persistent organic pollutants, as such and/or after metabolic activation, have been shown in in vivo or in vitro systems to possess endocrine-disrupting properties by interfering with estrogen, androgen, or thyroid hormones (6–9). Several compounds reported to be endocrine disrupters contain a phenolic group in their native form or as a result of metabolism. Examples include chlorinated phenols and hydroxylated metabolites of polychlorinated biphenyls (OH-PCBs). Both compound classes have been reported to be present in human blood and to have thyroidogenic properties (9–11). OH-PCBs have also been reported to compete for the estrogen receptor (12–14).

It is therefore of interest to determine the exposure of humans and wildlife to phenolic and neutral potential endocrine disruptors. Blood is a suitable sampling matrix as it is easily obtained from study subjects. Neutral lipophilic compounds are mainly distributed in the body depending on the lipid content in various tissues, and blood levels therefore often reflect the body burden of neutral lipophilic pollutants when determined on a lipid weight basis. However, less persistent compounds can also be determined in blood, and thus the recent exposure of short-lived, or semipersistent pollutants, such as phenolic OHS or metabolites, may be observed.

Lipophilic contaminants are transported in the blood by lipoproteins or proteins, such as albumin (15). Occasionally phenolic pollutants are retained in blood because of reversible but high affinity binding to specific proteins. Thus, chlorophenols and certain hydroxylated PCB metabolites have affinity for a thyroxin-transporting protein, transtyretine (16–18). The affinity of OH-PCBs for transthyretin may be 10 to 20 times higher than that of thyroxine (9,17). Thus, to enable extraction of such compounds, acidification and efficient denaturation of proteins is necessary.

Several methods for the analysis of contaminants in plasma have been reported (19–24). Traditional extraction methods, like those of Folch et al. (25) and Bligh and Dyer (26), are efficient in extracting lipid from various matrices including blood (25–27). However, most of the extraction and cleanup methods for OHS in blood samples are not specifically targeted to phenolic organohalogens, so low recoveries may be obtained. The aim of the present study was therefore to develop an efficient extraction method for blood plasma targeting both phenolic and neutral halogenated organic pollutants and to

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combine this with a nondestructive lipid removal method to allow for analysis of potentially unknown compounds. However, because of the simultaneous need for a rapid and evaluated method for analysis of OH-PCBs, three alternative lipid removal steps were studied: a high-performance liquid chromatography (HPLC)-based nondestructive procedure, a silica gel/sulfuric acid column, and a method using treatment with concentrated sulfuric acid (28). Depending on the choice of method for lipid removal, the described methods should allow either analysis of a broad range of environmental pollutants or rapid analysis of sulfuric acid stable compounds.

The compounds in this study are a selection of hydroxylated PCB congeners, halogenated phenols, and also some other pollutants present in environmental samples with varying chemical properties including PCB, DDE, trischlorophenyl methane (TCPM), trischlorophenyl methanol (TCPMeOH) (29,30), and bis-4-chlorophenyl sulphone (BCPS) (31). In addition, brominated flame retardants such as pentabromophenol (PBP) and tetrabromobisphenol A (TBBPA) and a few polybrominated diphenyl ether congeners (PBDE) were included (10). Finally, hexachlorocyclohexanes (HCHs) were included as examples of alkaline-sensitive pesticides.

Experimental

Chemicals

The compounds used, their abbreviation and source are listed in Table I. Two recovery (spiking) standards at different concentration were prepared for the neutral compounds and phenolic compounds, respectively (Table I). *n*-Hexane (Hx) and dichloromethane (DCM) (Fisons, Leicestershire, England) were of pesticide grade. Methyl tert-butyl ether (MTBE), 2-propanol, and potassium hydroxide (KOH) (Eka Nobel AB, Bohus, Sweden); methanol (MeOH), chloroform, potassium

Table I. Compounds Used in the Present Study and the Source and the Amounts Used*

Compounds	Low level (ng/sample)	High level (ng/sample)	Source
2,6-Dibromophenol (**diBP**)	140	600	Fluka AG, Buchs, Switzerland
2,4,6-Tribromophenol (**triBP**)	38	190	Fluka AG, Buchs, Switzerland
Pentabromophenol (**PBP**)	68	340	Aldrich Chemie, Steinheim, Germany
2,3,4-Trichlorophenol (**triCP**)	15	76	Janssen Chimica, Beerse, Belgium
2,3,4,5-Tetrachlorophenol (**tetraCP**)	17	160	Fluka AG, Buchs, Switzerland
Pentachlorophenol (**PCP**)	32	318	Kebo, Stockholm, Sweden (recrystallized)
Tetrabromobisphenol A (**TBBPA**)	77	120	Aldrich Chemical Co, Milwaukee, WI
Tris (4-chlorophenyl)methanol (**TCPMeOH**)	50	320	Larodan Fine Chemicals, Malmö, Sweden
Tris (4-chlorophenyl)methane (**TCPM**)	49	300	Larodan Fine Chemicals, Malmö, Sweden
Hexachlorobenzene (**HCB**) ø			Dr. Ehrenstorfer GmbH, Augsburg, Germany
α-Hexachlorocyclohexane (**α-HCH**)	31	200	Bellefonte, PA
γ-Hexachlorocyclohexane (**γ-HCH**)	31	200	Bellefonte, PA
1.1-di(4-chlorophenyl) 2,2-dichloroethene (**DDE**)	49	320	Bellefonte, PA
1.1-di(4-chlorophenyl)2,2,2-trichloroethane (**DDT**)	31	200	Bellefonte, PA
Bis (4-chlorophenyl) Sulfone (**BCPS**)†	22	110	Aldrich Chemical Co, Milwaukee, WI
2,2',3,6'-Tetrachlorobiphenyl (**CB-46**)†			Synthesized as described in reference 32
2,2',5,6'-Tetrachlorobiphenyl (**CB-53**)	22	110	Synthesized as described in reference 32
2,3,3',4,4',5,5'-Heptachlorobiphenyl (**CB-189**)	13	85	Synthesized as described in reference 32
2,2',3,3',4,5,5',6-Octachlorobiphenyl (**CB-198**)	13	84	Promochem AB, Ulricehamn, Sweden
2,3,6-Tribromodiphenyl ether (**BDE-30**)‡	22	110	Synthesized as described in reference 35
2,2',4,4'-Tetrabromodiphenyl ether (**BDE-47**)‡	20§	130§	Chemische Fabrik Kalk Köln,Germany
2,2',4,4',5-Pentabromodiphenyl ether (**BDE-99**)‡	22§	130§	Chemische Fabrik Kalk Köln,Germany
2,2',4,4',6-Pentabromodiphenyl ether (**BDE-100**)‡	3.4§	20§	Chemische Fabrik Kalk Köln,Germany
3-Hydroxy-2',5',6-triCB (**3'-OH-CB18**)‖	10	50	Synthesized as described in reference 36
4-Hydroxy-3,3',4',5-tetraCB (**4-OH-CB79**)#			Synthesized as described in reference 37
2-Hydroxy-2',3, 3',4,4'-pentaCB (**2'-OH-CB105**)‖	10	50	Synthesized as described in reference 36
4-Hydroxy-2, 2',3,3',4',5'-hexaCB (**4'-OH-CB129**)‖	10	50	Synthesized as described in reference 36
4-Hydroxy-2',3, 3',4',5,5'-hexaCB (**4'-OH-CB159**)‖	10	50	Synthesized as described in reference 36
2-Hydroxy-2',3, 3',4,4',5'-hexaCB (**2'-OH-CB156**)‖	10	50	Synthesized as described in reference 36
3-Hydroxy-2',3',4, 4',5,5'-hexaCB (**3'-OH-CB156**)‖	10	50	Synthesized as described in reference 36
4-Hydroxy-2,3,3',4',5,5',6 -heptaCB (**4-OH-CB193**)‖	10	50	Synthesized as described in reference 36

* Each sample was 5 g plasma.
† CB-46 was used as injection control standard
‡ Abbreviation according to PCB-numbering system (33).
§ Calculated amounts of the percentage occurrence of the individuals in the technical mixture, Bromkal 70-5DE (34).
‖ Abbreviation based on the PCB-numbering system (10).
\# 4-Hydroxy-3,3',4',5-tetraCB (4-OH-CB79) was ^{14}C-labelled with a specific activity of 26.5 Ci/mol. Used for fraction size determination in HR-GPC.

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chloride (KCl), and dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany); and H_2SO_4 (98%, w/w, BDH laboratory Supplies, Poole, England) were all of pro analysis quality. Ethanol (99.5%) was purchased from Kemetyl (Haninge, Sweden). Diazomethane was synthesized as described (38). Silica gel (< 0.063 mesh), purchased from Macherey-Nagel (Düren, Germany), was activated by heating it overnight at 280°C and allowing to cool to room temperature prior to use. Test tubes (16 × 150 mm and 13 × 100 mm) with Teflon-lined screw caps (Kimble) were supplied by Labora (Stockholm, Sweden). All glassware was heated at 300°C overnight prior to use.

Biological samples

Human plasma. Plasma was obtained from the blood donor laboratory at Danderyds Hospital (Stockholm, Sweden). The plasma was stored at −20°C.

Animals. Sprague Dawley rats (10 males, approximately 200 g) were dosed intravenously with ^{14}C-labelled 4-OH-3,3',4',5-tetrachlorobiphenyl (5 µ Ci/kg b.w.) dissolved in DMSO (100 µL/animal). At 4 h after dosing, the animals were sacrificed and the blood sampled. The blood samples were immediately separated into plasma and red blood cells by centrifugation (3000 rpm for 5 min).

Apparatus

Gas chromatography (GC) analysis was performed on a Varian 3400 GC, equipped with a Varian 8200 autosampler, an electron capture detector (ECD), and a split-splitless injector operated in the splitless mode. The column used was a DB5 column (30 m × 0.25-mm i.d., 0.25-µm film thickness, J&W Scientific, Folsom, CA) with hydrogen carrier gas and nitrogen makeup gas. The column temperature was held at 80°C for 2 min and then increased by 10°C/min to 300°C, which was held for 10 min. The injector temperature was 250°C, and the detector temperature was 360°C. The chromatographic data were recorded and processed by Elds Pro (Chromatography Data Systems AB, Kungshög, Sweden).

High-resolution gel permeation chromatography (HR-GPC) was performed on a Varian 9012 connected to a Varian 9050 UV-vis detector (Varian, Walnut Creek, CA) operated at 254 nm. Samples were injected via a Valco injector (Vici AG, Schenkon Switzerland) equipped with a 400-µL loop. Separation was performed on two serially coupled polystyrene-divinylbenzene copolymer columns (PL gel, 5 µ, 50 Å, 300 mm × 7.5-mm i.d., Polymer Laboratories, Shropshire, U.K.). The mobile phase consisted of Hx/DCM (70:30, v/v), and the flow rate was 1.0 mL/min. The chromatographic data were recorded as described.

Radioactivity measurements were performed on a Wallac 1409 scintillator (Wallac Oy, Turku, Finland). The scintillation cocktail used for lipophilic extracts was scintillator 299 (Packard) and for aqueous samples Optiphase II (Wallac Oy).

For evaporation of solvent volumes larger than 1 mL, a vacuum centrifuge was used (Genevac SF50, Sales Development Ltd., Ipswich, U.K.). For solvent volumes less than 1 mL, the solvent was evaporated under a gentle stream of nitrogen at approximately 30°C. Centrifugations were performed at 3000 rpm for 3–10 min on a table centrifuge (Wifug Ltd., Bradford, U.K.).

Methods

The method includes four steps as illustrated in Figure 1: (1) denaturation and extraction, (2) separation of phenolic and neutral compounds, (3) three alternative lipid removal steps, and (4) quantitation with gas chromatography (GC–ECD). The lipid extraction efficiency was determined by comparing lipid yields obtained by the described method with those obtained for five plasma samples extracted, with slight modification, as described by Bligh and Dyer (26). Briefly, plasma samples (5 g) were extracted using MeOH/chloroform (2:1, v/v, 19 mL) in a separator funnel (100 mL). Water (6.5 mL) and chloroform (6.5 mL) were added to the plasma sample to obtain a two-phase system. The organic phase was collected and the aqueous phase extracted once more with chloroform (12.5 mL). The two organic phases were combined, and after evaporation of the solvent, the lipid content was determined gravimetrically.

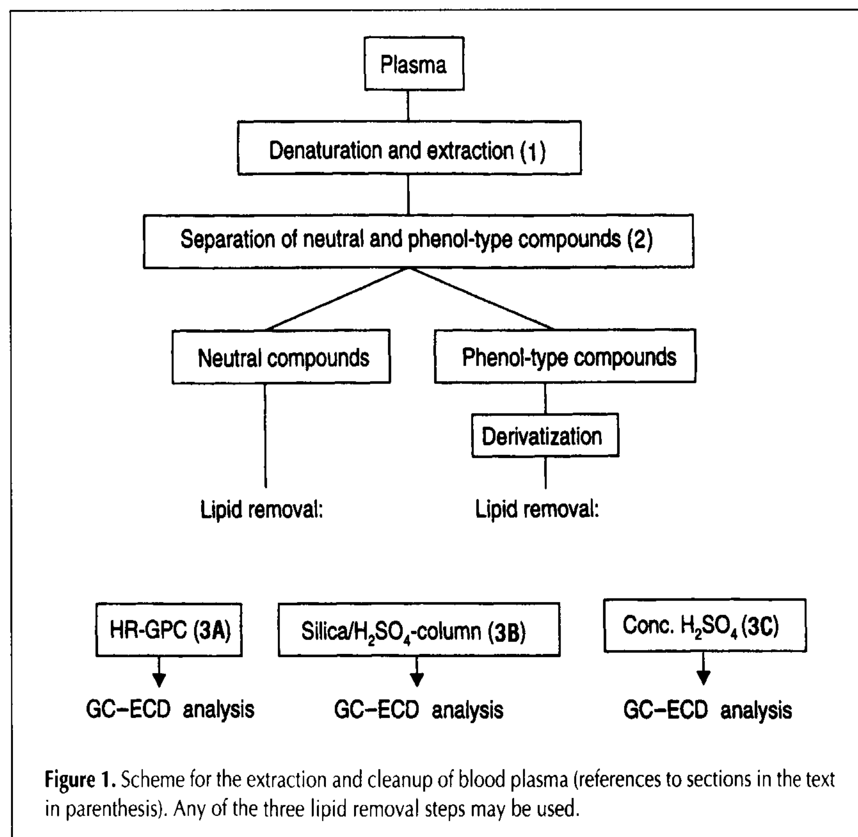

Figure 1. Scheme for the extraction and cleanup of blood plasma (references to sections in the text in parenthesis). Any of the three lipid removal steps may be used.

Denaturation and extraction (step 1). Plasma (5 g) was transferred to a screw-capped test tube. The recovery standards (total volume 100 µL; with compounds described in Table I) were added and the tube was mixed on a vortex mixer. HCl (6M, 1 mL)

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was added, and the contents were vortex mixed. 2-Propanol (6 mL) was added and the sample vortex mixed once more. Hx/MTBE (6 mL, 1:1, v/v) was added and the sample inverted for 5 min. After centrifugation, the organic phase was transferred to a second test tube, containing aqueous KCl-solution (1%, w/w, 4 mL). The denaturated plasma was re-extracted with Hx/MTBE (3 mL) and the organic phase combined with the first extract. Potentially co-extracted aqueous compounds in the extract were partitioned into the KCl-solution by gentle mixing. After centrifugation, the organic phase was transferred to a preweighed test tube. The aqueous phase was re-extracted with Hx/MTBE (3 mL) which was subsequently combined with the extract. The solvent was evaporated and the lipid content determined gravimetrically when constant weight was obtained.

Extraction efficiency. To determine the denaturation of proteins and the extraction efficiency for phenolic compounds, the plasma from all ^{14}C-labelled 4-OH-tetraCB administered rats were pooled, and the concentration of ^{14}C in the plasma was determined by scintillation counting. The pooled plasma was then divided into five samples (4 g) and extracted according to the method described here previously. The ^{14}C content was determined in the extract and in the remaining aqueous phase.

Separation of phenolic and neutral compounds (step 2). The extract, dissolved in Hx (4 mL), was partitioned with a KOH-solution (0.5M in 50% ethanol, 2 mL) and inverted 30 times, and after centrifugation, the organic phase was transferred to a test tube. The alkaline solution was re-extracted with Hx (3 mL) and the organic phases combined (the neutral fraction). The alkaline solution was acidified with HCl (2M, 0.5 mL) and phenolic compounds were extracted with Hx/MTBE (9:1, 4 mL) and subsequently extracted once more with 3 mL of the same solvent mixture. The organic phases were combined and represent the phenolic fraction. The solvent volume was reduced to approximately 1 mL. The phenolic and neutral fractions were treated separately in the subsequent steps.

Derivatization. Phenolic compounds were derivatized to their corresponding methyl ethers by the addition of ethereal diazomethane (0.5 mL, 3 h at 4–8°C) prior to any further steps. Ether and excess diazomethane were evaporated under a gentle flow of nitrogen at 30°C.

Lipid removal by HR-GPC (step 3A). The individual sample volumes were reduced to approximately 200 µL prior to injection into a 0.4-mL injection loop. The sample container was rinsed with 100 µL mobile phase that was also injected into the loop. Neutral compounds were obtained in a fraction collected between 18 min and 43 min, and phenolic compounds were obtained between 18 and 46 min. The fraction sizes were determined by the elution of HCB and the methyl derivative of TBBPA for the neutral compounds and of methyl derivatives of TBBPA and 2,6-diBP for the phenolic compounds. The individually collected fractions were concentrated to 1 mL and transferred to a dry silica-gel column (0.5 g) in a Pasteur-pipette, and the analytes were eluted with DCM (10 mL).

GC–ECD analysis requires exchange of DCM. In order to avoid losses, the solvent was concentrated to approximately 0.5 mL, an equal volume of hexane (0.5 mL) was added and the volume reduced to 0.5 mL. This procedure was repeated twice before GC–ECD analysis.

Lipid removal by silica/sulfuric acid gel column (step 3B). H_2SO_4 (diluted to 90% with water, w/w) was mixed with activated silica gel (1:2, w/w) by rotating the flask until no lumps were left. Impregnated gel (1 g) was transferred to a Pasteur-pipette that had been prepared with silylated glass wool and activated silica gel (0.1 g) in the bottom.

The extract of the neutral compounds was dissolved in DCM (approximately 0.5 mL) and transferred to the column. The compounds were eluted with 4 mL DCM, except for BCPS that required 28 mL DCM. In the present study, a 28-mL fraction was collected. The solvent was concentrated to approximately 0.5 mL, and the cleanup procedure was repeated once.

The extract of the derivatized phenolic compounds was dissolved in DCM (approximately 0.5 mL) and transferred to the sulfuric acid/silica gel column (1 g). The compounds were eluted with 10 mL DCM. The procedure was repeated but with half the amount of the gel (0.1 g SiO_2, 0.5 g SiO_2/H_2SO_4) and the compounds were eluted with 6 mL of DCM. For the GC analysis, the DCM was exchanged for hexane by the procedure previously described (step 3A).

Lipid removal by sulfuric acid treatment (step 3C). Each extract, dissolved in Hx (4 mL), was partitioned with concentrated H_2SO_4 (2 mL) by inverting the tube 20 times and then centrifuging it for approximately 5 min. The Hx-phase was transferred to a new test tube, and the H_2SO_4 phase was repartitioned with Hx (3 mL) and transferred to its respective test tube. After adjustment of sample volume, the derivatized phenolic compounds were analyzed by GC–ECD. The extract of the neutral compounds was concentrated (1 mL) and transferred to a column of silica gel/sulfuric acid (2:1, w/w, 0.5 g) in a Pasteur-pipette and eluted with Hx (10 mL).

Recovery experiment. The recovery standards, at high (HL) and low level (LL, Table I), were added to plasma samples: five replicate plasma samples of 5 g each for each concentration level. As control samples, five blank plasma replicates and two solvent blanks were used. In addition, LL and HL were added to a hexane solution and subsequently used as external references for the GC–ECD analysis. An injection control standard (CB-46) was added to all samples and controls prior to GC–ECD analysis.

Quantitation. The investigated compounds were quantified in spiked and unspiked samples and solvent samples. Recoveries were calculated by subtracting the levels determined in the unspiked plasma samples from those obtained in the spiked samples. For the substance recoveries, the arithmetic mean and the standard deviation were calculated.

Lipid determination. The lipid yield was determined gravimetrically. The mean value and the standard deviation (SD) of the lipid recovery results were calculated.

Results

The efficiency of the extraction method to extract a protein-bound OH-PCB from plasma was shown to be at least 98%. By

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using a radiolabelled compound, it was possible to determine the amounts of the compound in the plasma both prior to extraction and in the plasma extract without any cleanup steps where losses might occur. Well-separated and clear phases were obtained, and at least 98% of the radiolabelled material in the plasma was obtained in the extract. No radiolabelled material could be detected in the aqueous phase.

Lipid extraction from the human plasma (in total 30 parallel samples) was highly reproducible, with a coefficient of variation (CV) of ± 1.6% and a mean lipid content of 0.41%. The corresponding result for five parallel samples extracted with the modified Bligh and Dyer (26) method gave a variation coefficiency of ± 7.9% and a mean lipid content of 0.32%.

The overall recovery of the studied compounds at the two analyte levels, for all three different lipid removal methods (HR-GPC, the silica gel/H_2SO_4 column and partitioning with concentrated H_2SO_4), are presented in Tables II and III. The phenolic compounds generally gave a lower yield than the neutral compounds. In particular, the phenols with three or fewer halogen atoms gave low recoveries (approximately 40–60% compared to 80–90%) by all three methods. The low boiling point of these compounds most probably contributed to losses during evaporation steps, despite the precautions taken.

After using HR-GPC as the lipid-removal step, all compounds, phenolic and neutral, were recovered to varying degrees. The recoveries ranged from around 50–60% for the

Table II. Recovery (Mean and Standard Deviation) of the Neutral Analytes (Low and High Level) using the Different Lipid Removal Methods

	HR-GPC				SiO_2/H_2SO_4 column				H_2SO_4			
	Low		High		Low		High		Low		High	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
α-HCH	67	10	65	4	79	2	75	4	68	2	69	5
γ-HCH	72	6	69	3	77	2	79	4	74	2	72	5
CB-53	78	9	77	5	98	1	88	3	92	4	85	7
CB-189	92	3	88	3	95	4	98	2	92	1	89	6
CB-198	86	2	87	2	87	2	94	2	92	3	88	5
BDE-30	84	3	84	2	89	4	85	3	63	8	50	21
BDE-47	97	6	93	2	110	7	92	1	100	5	86	6
BDE-99	91	5	82	2	100	4	100	1	100	5	95	7
BDE-100	88	7	92	2	93	4	100	4	98	6	100	8
DDT	98	6	89	3	110	9	100	1	100	8	100	18
DDE	81	6	84	3	93	4	95	2	77	9	84	6
BCPS	95	3	83	3	110	8	90	3	0	0	0	0
TCPMe	90	7	88	5	96	2	112	3	77	3	72	6
TCPMeOH	85	6	86	5	0	0	0	0	0	0	0	0

Table III. Recovery (Mean and Standard Deviation) of the Phenolic Analytes (Low and High Level) using the Different Lipid Removal Methods

	HR-GPC				SiO_2/H_2SO_4 column				H_2SO_4			
	Low		High		Low		High		Low		High	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
2,6-DBP	41	10	43	4	44	3	53	8	30	5	35	3
2,3,4-TrCP	63	15	71	3	71	2	73	2	na*	na	na	na
2,4,6-TrBP	52	21	68	3	60	2	73	3	74	9	77	6
2,3,4,5-TeCP	69	15	76	3	69	3	78	0	na	na	na	na
PCP	68	15	72	3	63	6	78	2	73	8	81	8
3'-OH-CB18	78	17	80	3	130	6	96	2	110	12	100	9
PBP	80	19	82	2	80	3	92	3	90	5	86	8
2'-OH-CB105	78	18	78	2	87	3	90	3	90	3	87	8
4'-OH-CB129	78	19	75	2	83	3	89	3	76	6	82	8
4'-OH-CB159	74	18	76	2	80	3	89	2	79	4	80	6
2'-OH-CB156	82	18	82	2	91	3	93	2	89	5	88	7
5'-OH-CB156	77	18	78	2	86	3	91	2	85	5	85	7
4-OH-CB193	69	18	76	2	75	4	93	3	75	5	81	5
TBBPA	81	18	82	2	100	5	91	3	53	12	45	5

* na, not analyzed.

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low halogenated phenols, 75–82% for the OH-PCBs, to around 90% for most of the neutral compounds (Tables II and III). Concentrated sulfuric acid treatment resulted in recoveries ranging from a total loss of BCPS and TCPMeOH to almost quantitative recovery for some neutral compounds. TBBPA was poorly recovered (50%).

When the silica gel/sulfuric acid column was used for lipid removal TCPMeOH was lost, but the other compounds were recovered in high yields (Tables II and III). The recovery of the 3'-OH-CB18 was over 100%, indicating the presence of a co-eluting contaminant. However, no such corresponding peak could be detected in the blanks, and no explanation could be found for this error.

Discussion

In the present study methods for unbiased analyses of a broad spectrum of organic pollutants and for rapid analysis of primarily phenolic and neutral halogenated compounds in plasma have been evaluated. The same extraction and group separation steps are used, but depending on the purpose of the analysis, either nondestructive HR-GPC separation or silica gel/sulfuric acid column can be used. These methods were evaluated and compared with the classical treatment with concentrated sulfuric acid.

All three lipid-removal methods evaluated in the present study gave acceptable results for most compounds (Tables II and III), although a few compounds were lost when using the two methods with sulfuric acid. Thus, for the analysis of unknown substances the HR-GPC method is preferable because it is nondestructive. The separation mechanism for HR-GPC is mainly based on molecular volume of the analytes, but polarity and adsorption are also involved to some extent (39). HR-GPC retention times are therefore influenced by the chemical properties of both the analytes and the mobile phase. To avoid having overly polar analytes, the phenolic samples in this study were methylated prior to the HR-GPC column. Adsorption of phenolic compounds to glass surfaces can constitute a problem in the analysis. This risk is minimized by methylation of the phenolic fraction early in the cleanup procedure. By using methanol as solvent for reference and surrogate standards, and/or silylating the glassware, adsorption can be avoided.

The results in Tables II and III also show that all the studied compounds are recovered when using the HR-GPC method for lipid removal. The recovery for OH-PCBs ranged from 69 to 82 for both spiking levels and was slightly lower for the phenols. However, for TBBPA, 80% was recovered using HR-GPC, whereas only about 50% was recovered after the sulfuric acid treatment. No in-depth study was performed to trace the reason for the poor recovery. It may be speculated that the derivatized TBBPA was degraded by the concentrated sulfuric acid, as the recovery was high with the other two methods.

The recoveries for the phenols and the OH-PCBs were slightly higher when using the sulfuric acid or the silica gel/sulfuric acid column. The lower recovery after using HR-GPC may have been due to losses during the injection procedure. The need for exchanging the DCM-containing solvent prior to the GC-analysis may result in losses of volatile compounds during evaporation. For the phenolic compounds, the silica gel/sulfuric acid column gave the best results. None of the methylated phenolic compounds were lost using this method, and the recoveries were higher than with any of the other methods (Table III). In addition, the clean-up using sulfuric acid/silica is rapid, simple, and inexpensive.

All neutral halogenated compounds were recovered in good yields after using HR-GPC (Table II). Thus, none of the compounds were discriminated against using this method. Using sulfuric acid treatment, BCPS and TCPMeOH were lost, and TCPM was recovered in low yield. BCPS is a Lewis base as are the $MeSO_2$-PCBs and forms a complex with concentrated sulfuric acid. However, by diluting the sulfuric acid with one volume water compounds acting as Lewis bases can be re-extracted into the organic solvent (40). The TCPMeOH was lost also when using the silica gel/sulfuric acid column. The reason for this is not clear but presumably the compound is unstable in this environment, as it was not recovered after dilution of the concentrated acid.

DDT, DDE, and the PCB and PBDE congeners, except BDE-30, were all recovered in high yields with all the methods. The lower recovery of BDE-30 was observed after treatment with concentrated sulfuric acid, but the reason for this is not clear. To our knowledge, there have been no reports showing that brominated diphenyl ethers are labile upon acid treatment.

Regardless of the method, the HCHs were recovered at fairly low yields, ranging from 65 to 75%. Their high volatility and their lack of stability in alkali can partly explain the results. To minimize losses of HCHs in the present study, 0.5M KOH was used because this concentration of alkali was shown to be sufficient for isolation of halogenated phenols. Recently, 0.25M NaOH was shown to be sufficient for isolation of OH-PCBs (41) and thus might be used if HCHs are to be analyzed.

When analyzing persistent organic pollutants, the levels in biota are often normalized to the lipid content in the tissues studied. Lipid content is generally determined gravimetrically in tissues and often also for plasma. Method related differences in lipid yield cause discrepancies in results, even when the same amounts of analyte may be extracted. Lipid content determination and normalization has been discussed (27). In the present study, highly reproducible lipid yields were obtained and the yields were slightly higher than those obtained with the Bligh and Dyer (26) method that has often been cited as being the most efficient method for lipid extraction (27,42). No attempts to characterize the extracted lipids were performed in this study, and therefore the identity of the extracted material is unknown. Enzymatic lipid determination has been shown to correlate well with a Folch-type extraction method (43), although the latter gives a slightly lower yield than the former. The present method also correlates well ($r^2 = 0.85$) with the enzymatic lipid determination method and is published elsewhere (44).

In the sequence of steps included in a cleanup procedure, there are many potential places to lose the desired analytes. It was therefore convenient to study the extraction procedure efficacy for the protein-bound phenolic analytes by using a

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radiolabelled compound. The 4-OH-3,3',4',5-tetraCB is known to bind to a plasma protein, transthyretine (16), and by administrating it intravenously to rats, the real situation was mimicked. The radiolabelled compound could thus be quantitated in plasma prior to analysis as well as in the extract and in the aqueous phase without the need for clean-up steps.

The lipid-extraction and lipid-removal methods described in this report have different characteristics, but when considered as a set, these techniques can find utility in a range of OHS analytical problems. In the case of plasma samples, the desired extract can be further treated depending on the nature of the analytical problem such as for rapid determination of potential endocrine disruptors, particularly phenol-type OHS, for example, OH-PCBs and halogenated phenols. For neutral OHSs such as PCB, PBDE, and DDT/DDE, the sulfuric acid/silica gel column provides the highest recovery and can most easily be automated for high-throughput applications. The large number of unknown compounds, for example, natural products or degradation products from pharmaceuticals (45), require less discriminating cleanup methods, and thus HR-GPC is an appropriate solution.

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