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SPE-HPLC purification of endocrine disrupting compounds from human serum for assessment of xenoestrogenic activity

Abstract Assessment of xenoestrogenic activity in human serum samples requires the removal of endogenous sex hormones to assure that the activity measured originates from xenobiotic compounds only. Serum samples representing high, medium and lower accumulation of persistent organic pollutants (POPs) were extracted using solid-phase extraction (SPE) followed by normal-phase high-performance liquid chromatography (NP-HPLC) for separation of POPs from endogenous hormones. The recovery of polychlorinated biphenyl (PCB) congeners in spiked serum samples was up to 86 %, making the extraction method suitable for the study. MVLN cells, stably transfected with an estrogen receptor (ER) luciferase reporter vector (ERE-CALUX), were exposed to the reconstituted SPE-HPLC extracts for determination of the integrated estrogenic activity. The effects of PCBs were analyzed by direct *in vitro* exposure of PCBs (138, 153 and 180) and by *ex vivo* analysis of SPE-HPLC extracts from serum spiked with the PCBs. Similar effects on ER transactivation were observed for the direct *in vitro* and the *ex vivo* analysis experiments. The ER transactivation responses determined for actual serum samples were in the linear range of the dose-response curve. 17β-Estradiol titrations showed that the xenoestrogenic effects were mediated via ER. Moreover, our SPE-HPLC-ERE-CALUX assay was demonstrated to elicit high interlaboratory correlation. In the present study the combination of SPE-HPLC purification and the *ex vivo* estrogenic responses measured by ERE-CALUX was validated and considered to be a valuable tool to assess the combined ER effect of lipophilic serum POPs where additive/synergistic and agonistic/antagonistic effects are integrated giving an overall estimate of exposure and bioactivity.

Abbreviations BPA: Bisphenol A · BPA-DM: Bisphenol A dimethacrylate · CALUX: Chemically activated luciferase expression · CV: Coefficient of variation · DC-FCS: Dextran-treated fetal calf serum · DDE: Dichlorodiphenyl dichloroethylene · DDT: Dichlorodiphenyl trichloroethane · DMEM: Dulbecco's modified Eagle's medium · DMSO: Dimethyl sulfoxide · E1: Estrone · E2: 17β-Estradiol · EC_{40}: Concentration exerting 40 % of the effect of the maximal effective concentration · ER: Estrogen receptor · ERE: Estrogen response element · GC: Gas chromatography · HPLC: High-performance liquid chromatography · KHF: Female serum control · KHM: Male serum control · $\log K_{ow}$: Logarithm of the octanol–water partitioning coefficient · LTH: Laboratoire de la Toxicologie Humaine · MS: Mass spectrometry · NP: 4n-Nonylphenol · PCB: Polychlorinated biphenyl · POP: Persistent organic pollutant · SPE: Solid-phase extraction · t_R: Retention time · ε^o: Eluotropic strength · OP: 4-octylphenol

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Introduction

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and organochlor pesticides, including dichlorodiphenyl dichloroethylene (*p,p'*-DDE), have been reported to have a multitude of deleterious health effects on wildlife and humans, including dermal toxicity, immunotoxicity, carcinogenicity, and adverse effects on reproductive, neurobehavioral, and endocrine functions [1–8]. These compounds are lipophilic and degrade slowly

in the environment with long half-lives in organisms; for PCBs in blood the half-life is 1–10 years, depending on the congeners, and more than 10 years for *p,p'*-DDE [9–11]. These properties results in bioaccumulation and biomagnification through the food web [12, 13]. Indeed, POPs have been detected in food items, especially in fat tissues of marine animals, and also in human adipose tissue, breast milk, and blood [4, 14, 15].

It has become evident that several of the POPs have the potential to disturb the normal function of sex hormones in humans and animals. The xenoestrogenic potential of, e.g., PCBs and the insecticides dichlorodiphenyl trichloroethane (DDT) and dieldrin has been reported [16–19]. However, the overwhelming number of potentially endocrine disrupting substances produced makes it impossible to monitor the effect of every single compound within a reasonable time span. The distribution of POPs in tissues and body fluids has been thoroughly investigated and the results qualify serum samples to be well suited for exposure assessment since there is a good correlation between body burden and serum levels of accumulated POPs [20–22].

When dealing with environmental contamination of POPs and the assessment of its health impact on humans it is of great importance to measure the concentration of contaminants in the blood or tissues, which helps us to understand the extent and character of contamination, and it is an important tool in estimating correlations between exposure and health outcome. Nevertheless, it is of major interest to assess the actual xenobiotic activity on key receptor levels *in vitro* and *ex vivo* of the sum of interacting compounds found in exposed individuals, even if these compounds are present at very low concentrations. Individual activity assessment at these low physiological relevant concentrations *in vitro* might be below the detection limit. Although some POPs have the ability to interact with hormonal receptors, their chemical structures are not identical with those of hormones (Fig. 1), and generally POPs are more lipophilic than most hormones. This polarity difference can be exploited in a purification procedure. Human serum samples were subjected to a solid-phase extraction (SPE), wherein lipophilic substances (both POPs and hormones) were isolated from the serum matrix, followed by normal-phase high-performance liquid chromatography (HPLC) fractionation, which separated the POPs from the endogenous hormones [23–26].

The extraction of POPs from body fluids for determination of contaminant levels and/or for investigation of the interaction between contaminants and further assessment of xenoestrogenic bioactivity in cell culture models is a well-accepted approach. Earlier SPE-HPLC studies determined the estrogenic activities in human serum extracts using the E-screen method with the biological end point of proliferation of the human breast cancer MCF-7 cells [23–27]. The estrogen receptor (ER) transactivation assay, shown to respond to a wide range of different compounds with estrogen-like potentials [16, 27–30], is one of the basic tools used by others and in our laboratory to identify ER-mediated endocrine disrupters.
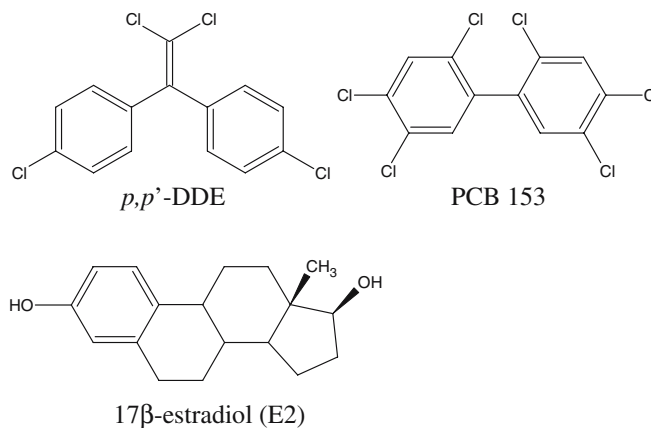

Fig. 1 Sample structures of compounds with endocrine-disrupting properties together with the female sex hormone 17β-estradiol for structural comparison

We have established and verified an SPE-HPLC method, with a few modifications compared with a previous report [26], for determination of xenoestrogenic activities in human serum using the MVLN cell line, carrying a stable integrated ER reporter gene (estrogen response element, ERE, luciferase) [31]. *In vitro* direct exposure and *ex vivo* analysis of SPE-HPLC extracts of PCB-spiked serum as well as of actual serum samples showed similar ER transactivation responses. Moreover, our data were in accordance with those obtained for samples previously extracted by SPE-HPLC in another laboratory.

Materials and methods

Reagents

All solvents used were high-purity grade for HPLC: *n*-hexane, ethyl acetate, methanol, 2-propanol, acetone (Fisher Scientific, Leicestershire, UK). Ethanol 96 % (extra pure was from Merck (Darmstadt, Germany). 17α-Hydroxypregnenolone, pregnenolone, progesterone, testosterone, androsterone, estriol, and estrone (E1) were from Steraloids (Newport, RI, USA). 17β-Estradiol (E2), 17α-ethinyl estradiol, 4-octylphenol (OP), bisphenol A (BPA), and BPA dimethacrylate (BPA-DM) were from Sigma-Aldrich (St. Louis, MO, USA). 4-Hydroxy-2′,3,4′,5,6′-pentachlorobiphenyl was from AccuStandard (New Haven, CT, USA). PCB 81, PCB 126, PCB 138, PCB 153, PCB 180, endosulfan, vinclozolin, methiocarb, fenarimol, and prochloraz were from Dr. Ehrenstorfer (Augsburg, Germany). 4*n*-Nonylphenol (NP) was from Lancaster Synthesis (Lancaster, UK). Butylated hydroxyanisole was from ICN Biomedicals (Aurora, OH, USA).

The stock ethanolic solutions (ethanol 96 % extra pure) of all calibrators, test compounds, and internal standards were prepared once and stored at 5 °C in brown vials with screw caps and sealed with parafilm. The working standards were prepared every day by appropriate dilution of the stock solutions with *n*-hexane or methanol.

Instrumentation

Solid-phase extraction

SPE was carried out using Oasis HLB extraction cartridges (6 ml, 500 mg, Waters, Milford, MA, USA). Extracted compounds were collected using a VAC ELUT SPS 24 vacuum manifold (Varian, Harbor City, CA, USA). POPs were extracted from the serum samples by a modification of an SPE method previously described [23, 26]. Briefly, the cartridges were washed with 4 ml *n*-hexane and 3 ml ethyl acetate to remove potential contaminations. After conditioning [23], 3.6 ml serum was applied. To remove proteins the cartridge was subsequently washed with 4 ml 5 % acetone in water (v/v) and dried by vacuum. The POPs were eluted with 4 ml methanol followed by 4 ml ethyl acetate drawn through by gravity. Working up the eluate after liquid–liquid extraction as described [23], we reduced the pooled supernatants to less than 1 ml by vacuum, and the final volume was reduced to near dryness under N_2 at 31 °C using a TurboVap LV (Zymark, Hopkinton, MA, USA). The extract was reconstituted in 315 µl *n*-hexane–ethyl acetate (9:1, v/v) and transferred to Total Recovery vials (Waters, Milford, MA, USA), from which 300 µl was injected into the HPLC system.

High-performance liquid chromatography

The HPLC fractionation was performed using an Alliance 2695 separations module with a 300 µl injection loop, equipped with a 2996 photodiode array detector, a Fraction Collector II (Waters, Milford, MA, USA) and a Spherisorb Si 60 analytical column (250 mm × 4.6 mm inner diameter, 5 µm, Waters, Milford, MA, USA) with chromatographic parameters similar to those in [23]. The eluate was collected in fractions defined on a time scale (Table 1). All fractions were evaporated under N_2 at 31 °C and stored at −80 °C until they were analyzed in the ERE chemically activated luciferase expression (CALUX) bioassay. Extracts corresponding to specific fractions were named accordingly, i.e., F1 for fraction 1 and F2.1 for fraction 2.1, and so on.

Retention time (t_R) drift was monitored by injecting a test sample before each sample set and recording the difference in retention over time. The test sample consisted of PCB 81, progesterone, E1, and testosterone, 20, 50, 200, and 50 µM, respectively, dissolved in *n*-hexane (Fig. 2). Four independent procedural blanks were processed once to determine if contamination occurred originating from the materials used, e.g., from solvents, SPE cartridges or instruments. Water (3.6 ml ultrapure ELGA) was extracted instead of serum in the SPE-HPLC procedure that was otherwise followed exactly as described in "Solid-phase extraction" and "High-performance liquid chromatography." The final SPE-HPLC extract was evaporated under N_2 at 31 °C.

Table 1 Retention times for persistent organic pollutants (*POPs*) and endogenous hormones

Compounds	t_R (min)	$\log K_{ow}$
Fraction 1	0.00–5.30	
PCB 180	1.98	7.1[c]
PCB 126	2.05	6.3[c]
PCB 81	2.08	5.9[c]
Endosulfan	2.16	3.83[a]
PCB 153	2.37	6.7[c]
PCB 138	2.49	6.7[c]
BPA-DM	2.56	NA
o,p′-DDT	2.66	6.79[b]
4*n*-Nonylphenol	2.73	5.76[b]
4-Octylphenol	2.74	4.12[d]
4-OH-PCB 121	2.75	NA
Vinclozolin	2.83	3.03[a]
Endosulfan	3.04	3.83[a]
BPA	3.11	3.32[b]
BHA	4.54	3.50[b]
Methiocarb	4.59	2.92[a]
Fraction 2.1	5.30–12.00	
Pregnenolone	5.79	4.22[b]
Androsterone	6.41	3,69[b]
Progesterone	6.61	3.87[b]
Fenarimol	6.85	3.69[a]
Estrone	8.54	3.13[b]
Prochloraz	9.41	4.10[a]
Testosterone	10.27	3.32[b]
Fraction 2.2	12.00–14.50	
17β-Estradiol	13.72	4.01[b]
Fraction 2.3	14.50–22.00	
17α-Ethinyl estradiol	16.34	3.67[b]
Fraction 2.4	22.00–30.00	
Estriol	23.55	2.45[b]

$\log K_{ow}$ values for reference
[a][37], [b][38], [c][39], [d][40]

Gas chromatography–mass spectrometry

Gas chromatography (GC) was performed using a Hewlett-Packard 6890 gas chromatograph equipped with a Hewlett-Packard 5973 mass spectrometer. The separation was performed on an HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) with cross-linked (5 % phenyl)-methylpolysiloxane. The autoinjector was a Hewlett-Packard 7683. Separation was achieved by pulsed splitless injection with an inlet temperature of 250 °C. The program was as follows: initial temperature 90 °C (5 min); 15 °C/min to 230 °C for 20 min. The carrier gas was helium at a flow of 1.3 ml/min. The injection volume was 2 µl, and samples were dissolved in *n*-hexane–ethyl acetate (9:1, v/v). The samples were ionized with electron impact with an ionizing energy of 50 eV and an emission current of 100 µA. The following ions were detected in single ion monitoring mode: *m/z* 290, 292, 326, 328, 360, and 362.
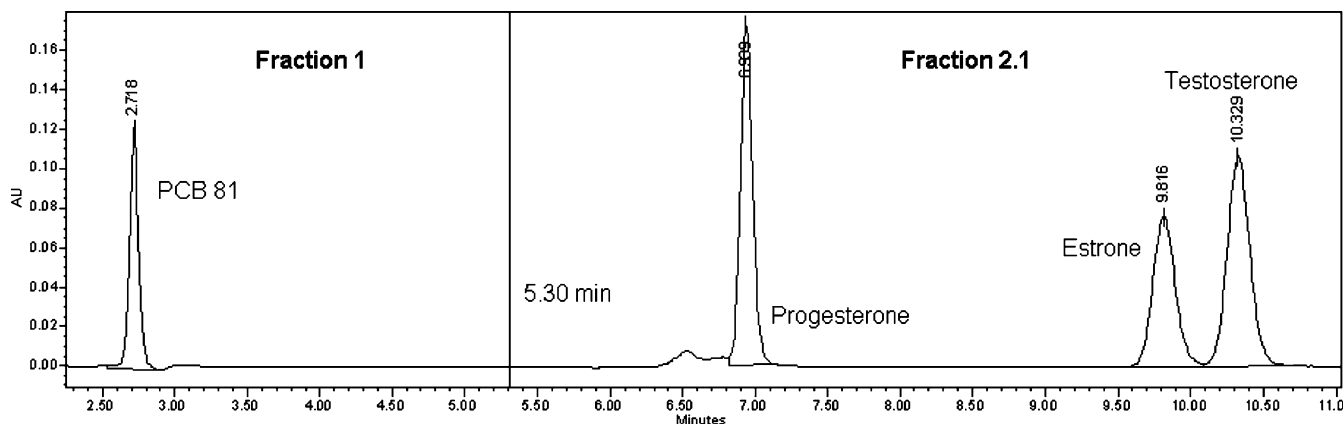
Fig. 2 Retention time control. A mixture of four compounds with varying polarity (PCB 81, progesterone, estrone, testosterone) was injected before each sample set to monitor retention drift

Serum controls and serum samples

Human serum from various sources was collected for two purposes: firstly, to evaluate the extraction efficiency of the SPE-HPLC method by performing a series of recovery experiments and, secondly, to evaluate the overall performance over time of the entire assay. A set of serum control samples was established by pooling two batches of male serum control (KHM) and two batches of female serum control (KHF), respectively, (kindly donated by the blood bank at Aarhus Sygehus, Aarhus, Denmark), dividing the pools into 4 ml aliquots in Cryo Tubes (Nunc, Roskilde, Denmark), and storing them at −80 °C.

Recovery experiments were performed with serum from four different sources:

1. From young Danish male volunteers by drawing blood in 4 ml plain Venoject tubes (Teruno Europe, Leuven, Belgium). The blood samples were then left at ambient temperature for 2–3 h for the blood to clot before being centrifuged at 3,300*g* for 12 min. The top layer of serum was transferred to Cryo Tubes and was stored at −80 °C.
2. Swedish male serum residues pooled from a batch of project samples [28].
3. Serum from the stock of KHM and KHF samples.
4. Serum residues from Inuits.

To monitor the overall performance of the entire assay one of each KHM and KHF sample was extracted by SPE-HPLC and evaluated in the ERE-CALUX assay each week in parallel with the serum test samples. Spiked KHM samples and pooled residuals of Swedish serum (Table 2) were used for analysis in the ERE-CALUX assay to estimate SPE-HPLC extraction efficiency.

Spiking experiments and SPE-HPLC recovery

Recovery was determined in both actual test serum samples, control serum samples, and in spiked serum control samples (KHM and KHF). Serum samples were extracted by SPE-HPLC and evaporated. The almost-dry SPE-HPLC extract and the originating serum were sent to a contract laboratory (Laboratoire de la Toxicologie Humaine (LTH), Sainte-Foy, QC, Canada), and both samples were analyzed for POPs (Table 3) by gas chromatography–mass spectrometry (GC-MS). The recovery was calculated as the ratio of the POP content in the extract to the POP content in the serum. Spiked serum samples were prepared by adding ethanolic solutions of *o,p*′-DDT, BPA-DM, NP, PCB 81, PCB 126, and PCB 180 to human control serum (KHM and KHF) followed by brief vortexing (Table 4). The spiked serum was left at ambient temperature for 2 h before being extracted by SPE-HPLC. The almost-dry extract was redissolved in 2×200 μl *n*-hexane.

To estimate the efficiency of the extraction procedure, the ERE-CALUX activities of the three PCBs 138, 153 and 180 were determined *in vitro* directly and after extraction by SPE-HPLC of spiked serum control samples (KHM and KHF). Since the sum of the concentrations of PCB 138,

Table 2 PCB levels in spiked serum control samples (KHM) for SPE-HPLC-ERE-CALUX analysis

Compound	M_r	PCB spiked (μg/l)[a]		PCB spiked (μg/l)[a]			Pooled serum/POPs (μg/l)[b]		
		Mix1	Mix2	Low	Mean	Max	Low	Medium	High
PCB 138	360.88	3.3	6.6	1.72	8.3	77.8			
PCB 153	360.88	4.8	9.6	2.49	12.0	112.7	0.7	1.1	1.4
PCB 180	395.33	4.9	9.8	1.72	8.3	77.8			

[a]The spiked PCB concentrations mimic the levels of the total PCB burden measured in the AMAP '97 study [32]
[b]Pooled Swedish serum residues [28], classified after PCB 153 content but the samples contain many other compounds, which may also exert an estrogenic action

PCB 153, and PCB 180 is approximately half the total burden of PCBs in humans, these "physiological" PCB concentrations were multiplied by 2 in the spiking solution, thereby simulating an approximate total PCB burden in Greenlandic Inuit serum [32]. Spiking of PCBs was done in duplicate and in three different concentrations: low, mean and maximum, where low and maximum are the lowest and highest concentrations of the three PCBs measured, respectively (Table 2).

Dissolution of SPE-HPLC extracts for ERE-CALUX analysis

The SPE-HPLC extracts (project samples and controls) were thawed and protected from light during handling. Sample solvent, 20 µl EtOH:H_2O:DMSO (50:40:10, v/v/v), was added to each sample tube and the samples were placed in an Eppendorf Thermomixer Comfort at 550 rpm and 37 °C for 15 min. Then 200 µl growth medium [phenol red free Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Denmark) supplemented with 4 mM glutamine (Sigma-Aldrich, Denmark) and 1.28 mg/ml garamycin (Schering-Plough, Dassel, Germany) and 0.5 % dextran–charcoal treated fetal calf serum (DC-FCS, Hyclone, Bie & Berntsen, Aarhus, Denmark)] was added to each sample, and shaking at 550 rpm at 37 °C was continued for another 15 min. Then each sample was transferred to two new test tubes (100 µl per tube) each containing 400 µl growth medium with and without the normal ER ligand E2, respectively. These samples were used in triplicate (100 µl per well) for ERE-CALUX determinations.

Table 4 Recovery of POPs from spiked control serum samples (KHM) in fraction F1 after SPE-HPLC separation

Compound	HPLC		GC-MS	
	Recovery (%)	n	Recovery (%)	n
o,*p*-DDT	54	2		
BPA-DM	82	2		
4*n*-Nonylphenol	53	2		
PCB 81			86	3
PCB 126			58	2
PCB 180			36	2

Determined in our laboratory by HPLC or GC-MS. Recoveries were determined as the ratio of the peak area from the spiked compound to the peak area from a pure standard

n number of analyzed samples

ERE-CALUX assay

The stable transfected MVLN cell line (kindly provided by M. Pons, France) carrying the ERE luciferase reporter vector [29, 31] was used for determination of the ER transactivation response as described earlier [28, 33]. Briefly, MVLN cells were seeded in 96-well culture plates at a density of 3×10^4 cells per well and cultured in phenol red free DMEM containing 1 % DC-FCS for 24 h, and after removal of the medium exposed to the chemicals or serum extracts (see "Dissolution of SPE-HPLC extracts for ERE-CALUX analysis"), in 100 µl per well. After incubation for another 24 h and removal of the medium, the cells were lysed and luciferase activity was measured in a LUMIstar luminometer (BMG, RAMCON, Denmark) and the protein content was determined by fluorometric measurements with a WALLAC VICTOR2 (PerkinElmer, USA) as described in [33]. The luciferase data are given as relative light units per microgram (RLU/µg) of protein. SPE-HPLC

Table 3 Recovery of POPs from unspiked serum samples in fraction F1 after SPE-HPLC separation

Compound	Danish male volunteers			KHM + KHF			Pooled Swedish serum residues [28]			Detection limit (µg/l)
	Recovery (%)	n	Level found (µg/l)	Recovery (%)	n	Level found (µg/l)	Recovery (%)	n	Level found (µg/l)	
PCB 99	69	5	0.05							0.02
PCB 118	79	6	0.05	69	7	0.02				0.02
PCB 138	39	7	0.18	37	8	0.10	45	1	1.15	0.02
PCB 153	38	7	0.41	32	8	0.10	46	1	2.65	0.02
PCB 156	39	6	0.04							0.02
PCB 170	28	7	0.10	25	3	0.04				0.02
PCB 180	25	7	0.29	21	7	0.10	31	1	1.90	0.02
p,*p*′-DDE	45	7	1.75	43	8	0.30				0.05
p,*p*′-DDT	38	1	0.31							0.05
HCB				43	7	0.10				0.02
β-HCH	54	2	0.23							0.05
Oxychlordane	63	4	0.03							0.02
trans-Nonachlor	47	2	0.05							0.02

Recovery was determined by GC-MS at LTH, Canada

n number of samples analyzed

F1 extracts of the serum controls (KHM and KHF) were analyzed weekly in parallel. The solvent controls consisted of 20 µl solvent (EtOH:H$_2$O:DMSO, 50:40:10, v/v/v) and medium (with and without added E2). The solvent controls were handled and analyzed the same way as the SPE-HPLC serum extracts.

In each independent experiment, the concentration response of E2 (0.05–300 pM) was determined in parallel. The E2 concentration of 25 pM, exerting 40 % of the effect of the maximal effective concentration (EC$_{40}$), was used to determine the competitive xenoestrogenic effect of F1 serum extracts upon coexposure of the MVLN cells, and was analyzed in parallel on each plate as a positive control. The average ERE-CALUX inter-coefficient of variation (CV) of the solvent controls was less than 5 % and the CV of the response to E2-EC$_{40}$ was less than 15 %.

Calculations and statistical analysis

The data for each assay were related to the solvent control, which was set to 100 %, and are presented as the means ± the standard deviation. The statistical significance was evaluated by Microsoft Excel using the F test (variance homology) and Student's t test according to equal or unequal variance between the groups. The criterion for statistical significance was set to $p < 0.05$. EC$_{40}$, EC$_{50}$, and EC$_{100}$ calculations for E2 were performed by SigmaPlot 8.0 (SPSS, Chicago, Il, USA) using the sigmoid Hill model as described in [28, 33]. The association between the xenoestrogenic activities of the different SPE-HPLC fractions was evaluated by means of Spearman's rank correlation using the STATA program Intercooled Stata 9.

Results

Determination of time windows for HPLC fraction collection

The retention times for a broad range of compounds (Table 1) were determined to identify how to split the HPLC fractions to separate POPs from endogenous hormones. To avoid any overlap with endogenous hormones, fraction 1 (F1) was discontinued at 5.30 min. Within this time span most POPs had been eluted. The next fraction (F2.1) continued from 5.30 to 12.00 min followed by F2.2 ending at 14.50 min. Fraction F2.1 and F2.2 contained mostly endogenous hormones but also a few medium polarity pesticides. It was not possible to optimize the system to separate POPs from hormones when they had similar solubility properties. The solubility properties of POPs rely to some extent on the solubility of the compounds in the stationary phase of the HPLC column and can, to a certain extent, be quantified by the logarithm of the octanol-water partitioning coefficient (logK_{ow}). The value of logK_{ow} drops as the polarity of the corresponding compound rises and high values are associated with lower t_R on the normal-phase column.

Large volumes of sample solvent (i.e., 300 µl) can change t_R of the injected compounds especially if compared with the 5–20 µl often used in analytic chromatography. To verify t_R changes seven compounds were individually injected in increasing injection volumes (20, 50, 100, 150, 250 µl). By introducing the factor $\Delta t_R = t_{R,250µl} - t_{R,20µl}$ (subtracting t_R of the 250 µl injection from t_R of the 20 µl injection) the variation in t_R can be estimated. Figure 3 shows that all compounds have a positive Δt_R, indicating that they had a higher t_R when injected in 250 µl. While PCB 81 has a Δt_R of approximately 1 s, the hormones have values many times higher and it is hence plausible to conclude that nonpolar compounds are affected very little by the volume of the injection solvent in this experiment. The change in t_R indicates that the eluotropic strength (ε^o) of the mobile phase was temporarily changed towards a less polar action following injection, resulting in a slower motion of the more polar compounds. Calculation of the eluotropic strength [34] for the mobile phase ($\varepsilon^o = 0.041$–0.051) and for the injection solvent ($\varepsilon^o = 0.038$–0.057) revealed that the elution power was almost identical for the two solvent compositions. In order to explain the observed behavior in retention shift the lower estimate of ε^o ($\varepsilon^o \approx 0.038$) for the injection solvent must be assumed.

From this experiment, the t_R values shown in Table 1 were determined using the same volume of injection solvent as used for the SPE-HPLC extracts, namely, 300 µl. The test samples were likewise dissolved in n-hexane–EtOAc (9:1, v/v).

Retention time drift

No noteworthy drift in retention time was observed for PCB 81 over a period of several months (Fig. 2). Some deviations were observed for E1 and testosterone, indicating that the more polar compounds are sensitive to variations in the active sites on the sorbent bed. These variations could be due to inactivation of silica hydroxyl groups caused by moisture in the samples or in the mobile phase. Since the nonpolar compounds are almost unaffected by these variations, no precautions had to be taken.

Test for procedural blank contamination

The final dry procedural blank SPE-HPLC extract was analyzed by GC-MS (LTH, Canada) for traces of 31 different organochlorine residues. The list of compounds includes 14 PCBs and Aroclor 1260, 11 chlorinated pesticides, including DDT, DDE, aldrin, hexachlorobenzene and Mirex, and five toxaphenes (Parlar 26, 32, 50, 62, 69). None of the compounds was detected in these procedural blank extracts. This result demonstrates that our method is clean and that the compounds found in the extracts originate from the serum samples only.
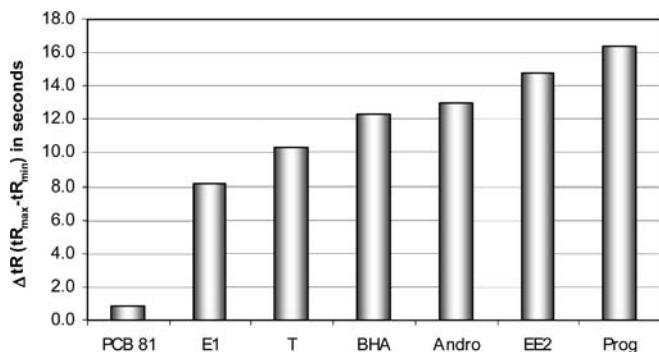
Fig. 3 Maximum retention time variation with a large volume of injection solvent. Injection of test compounds in large volumes of the injection solvent slows down elution, causing t_R to increase, thereby giving high Δt_R values. Little variation is seen for PCB 81, while great variation is seen for the hormones. The compounds belong to the following fractions (see Table 1): fraction 1 PCB 81, butylated hydroxyanisole (*BHA*); fraction 2.1 estrone (*E1*), testosterone (*T*), androsterone (*Andro*), progesterone (*Prog*); fraction 2.3 17α-ethinyl estradiol (*EE2*)

SPE-HPLC recovery

The unspiked SPE-HPLC recoveries of serum samples determined by LTH, Canada, were in the range from 21 to 79 %. Several of the environmental compounds detected in the samples were close to the detection limit (Table 3). The recoveries from the spiked samples, on the other hand, ranged from 36 to 86 % (Table 4). Both HPLC-UV detection and GC-MS detection were applied when analyzing the spiked serum samples. Because of elution of a broad unknown peak early in the chromatogram with UV detection, it was difficult to determine the peak area of the PCBs using HPLC. Furthermore, the retention times of these PCBs were very similar and they were therefore hard to distinguish. Because of these conditions, GC-MS was chosen for recovery determination in the PCB spiking experiments. Peak heights and retention times were readily observed for *o,p′*-DDT, BPA-DM, and NP on the HPLC system.

ERE-CALUX analysis

The ERE-CALUX assay demonstrated a dose-dependent increase in luciferase activation upon exposure to E2 in the concentration range 5–300 pM, and the maximum and the half-maximum effective concentrations (EC_{100} and EC_{50}) were reached at 150 and 33 pM E2, respectively, as previously reported [33].

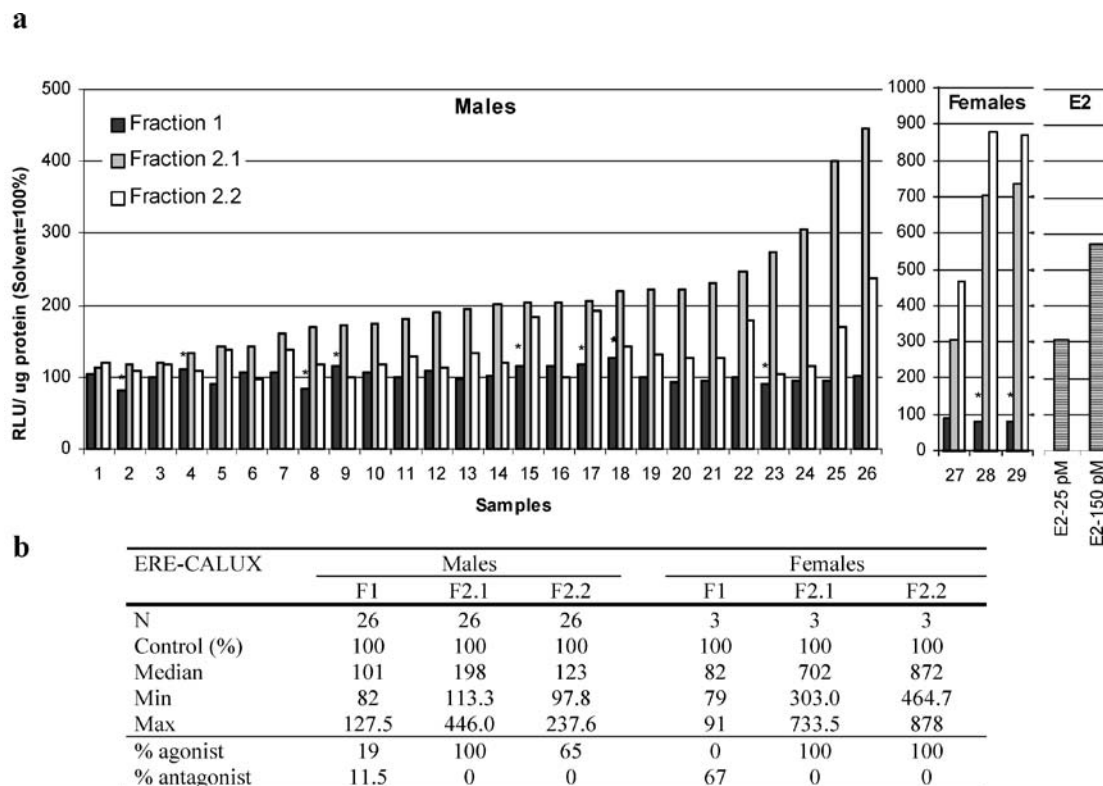| ERE-CALUX	Males			Females		
	F1	F2.1	F2.2	F1	F2.1	F2.2
N	26	26	26	3	3	3
Control (%)	100	100	100	100	100	100
Median	101	198	123	82	702	872
Min	82	113.3	97.8	79	303.0	464.7
Max	127.5	446.0	237.6	91	733.5	878
% agonist	19	100	65	0	100	100
% antagonist	11.5	0	0	67	0	0

Fig. 4 Estrogenic activities of SPE-HPLC fractions *F1*, *F2.1* and *F2.2* of human serum samples. **a** Serum samples from men (*1–26*) and women (*27–29*) were extracted by SPE-HPLC, and HPLC fractions 1, 2.1, and 2.2 were analyzed in parallel in the ERE-CALUX assay. For fraction *F1*: **p<0.05* is statistically significant from the solvent (100 %). **b** *N* is the number of serum extracts, *control* indicates the ERE-CALUX activity in the corresponding solvent control (background), *median*, *min*, and *max* refer to the ERE-CALUX activity, and *% agonist* and *% antagonist* indicate the percentage of samples exhibiting a significant (*p<0.05*) increase or decrease in activity compared with the corresponding solvent control, respectively. The relative light unit (*RLU*) normalized to cell protein (RLU per microgram of protein) of the solvent control was set to 100 %

Estrogenic activity of HPLC fractions from the separation steps

In order to confirm the removal of the endogenous hormones from the lipophilic POPs, the different fractions (F1, F2.1, and F2.2) from the HPLC cleanup procedure were analyzed for estrogenic activity. Serum residues from males (Sweden and Poland [28]) and Greenlandic Inuit female volunteers were extracted by SPE-HPLC and the different fractions were analyzed in parallel in MVLN cells. As shown in Fig. 4a, the main estrogenic activity was measured in F2.1 and F2.2, which is designed to contain the endogenous hormones in contrast to F1, which includes the lipophilic POPs only. All of the F2.1 and 65.5 % of the F2.2 extracts significantly ($p < 0.05$) increased the luciferase expression above the background solvent control (Fig. 4b). The maximum estrogenic responses in F2.1 extracts were 4.5-fold and 8.8-fold above that of the solvent control for male and female extracts, respectively. However, the maximum induction observed for E2 (150 pM) in the same assay was only sixfold above that of the solvent control. The median estrogenic responses in F2.2 extracts were 8 times greater in female than in male samples, being 8.7-fold and 1.2-fold above that of the solvent control, respectively.

A positive correlation was found between the estrogenic activities in F2.1 and F2.2 (STATA, Spearman's $r_s = 0.55$, $p = 0.002$), whereas no correlation was found between the estrogenic activities in F1 and F2.

Compared with F2, the corresponding F1 of all the extracts did not show the same level of estrogenicity (Fig. 4). In contrast to F2 only 17 % of all the F1 extracts significantly increased the luciferase activity, and 17 % decreased the ER-mediated activity. The maximum effect of F1 observed was 1.3-fold above the background.

In vitro effect of PCBs on ER transactivation: direct exposure of MVLN cells

Previously, we reported [16] that by transient transfection of MCF-7 cells with commercial PCB 138, PCB 153, and PCB 180 individually or in a mixture exhibit antiestrogenic effects on ER-mediated transactivation of a reporter gene expression. In the present study the antiestrogenic effect of the three PCB congeners alone and in a mixture was assessed by exposing the MVLN cells to PCBs alone or in the presence of 25 pM E2 (EC_{40}) (Fig. 5). The concentrations of PCB 138, PCB 153, and PCB 180 in the culture medium were 7.6, 11.1, and 6.9 μM, respectively. Each of the three PCBs significantly inhibited the basal and the 25 pM E2-induced luciferase activity in the undiluted samples. PCB 138, PCB 153, and PCB 180 significantly reduced the basal and E2-induced luciferase activity by 25 and 38 %, 12 and 19 %, and 30 and 13 %, respectively (Fig. 5).

Exposure of the MVLN cells to a mixture of all three PCBs at the concentrations given (legend to Fig. 5) significantly reduced the basal luciferase expression by 46 % relative to the solvent control. Interestingly, upon cotreatment with 25 pM E2, this mixture repressed the E2-induced luciferase activity by 55 % of the level for E2 alone, reaching the basal solvent level (Fig. 5).

Furthermore, we examined whether the antiestrogenic effect of individual PCBs as well as that of the mixture could be counteracted by dilution of the samples 5 and 10 times. The ER repressing effect of each individual PCB was abolished by dilution, whereas the effect of the mixtures in the absence or the presence of E2-EC_{40} could not be counteracted by either 5 times or 10 times dilution of the samples. Although the inhibiting effect of the PCB mixture on the luciferase activity was not counteracted by dilution, the repressing effect was reduced in a dose-
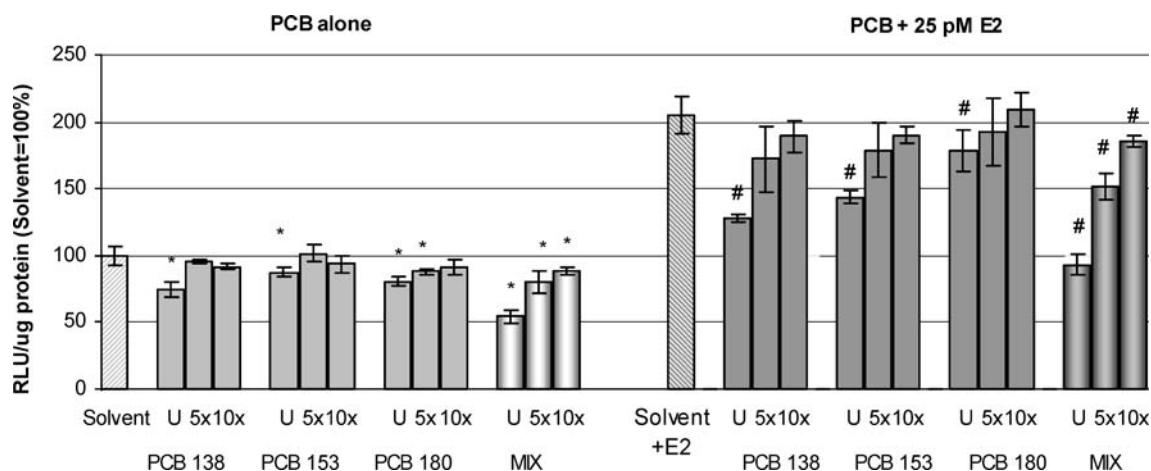

Fig. 5 Effects of PCB congeners on ER mediated transactivation. MVLN cells were exposed to PCB at given concentrations (PCB 138 7.6 μM, PCB 153 11.1 μM, PCB 180 6.9 μM and mixture, *MIX*, 7.6+11.1+ 6.9 μM), undiluted (*U*) and after 5 times (*5×*) and 10 times (*10×*) dilution alone or in the presence of 25 nM 17β-estradiol (*E2*). The RLU per microgram of protein (see legend to Fig. 4) of the solvent control (0.1 % ethanol) was set to 100 %. Data are expressed as the mean ± the standard deviation. * and # statistically significant ($p < 0.05$) difference from solvent control or 25 nM E2 reference control, respectively

dependent manner (linear regression, in the absence or presence of E2, $R^2=0.91$ and $R^2=0.98$, respectively).

Dose-response range of F1 serum extracts with low and high levels of PCB 153

In order to determine the dose-response range of F1 serum extracts, pooled residuals of serum samples [28] with low, medium and high concentrations of PCB 153 (Table 2) were extracted and further analyzed in the ERE-CALUX assay. MVLN cells were exposed to various volumes of the reconstituted F1 extracts in the presence and in the absence of E2. No statistically significant (anti)estrogenic effect was found in the absence of E2 (data not shown). Upon coexposure with E2-EC$_{40}$, increasing ER activity was observed with increasing volumes of F1 extracts (Fig. 6), and linear regression analysis showed a positive linear trend (linear regression, low, medium, high: $R^2=0.98$, $\alpha=0.0799$; $R^2=0.48$, $\alpha=0.0277$; $R^2=0.73$, $\alpha=0.0605$, respectively). These results indicated that the response of the different POP levels in the present serum samples was in the linear dose-response range.

As a day-to-day control, an F1 extract from serum control samples (KHF and KHM) was analyzed in parallel, and the inter CV of these samples in the ERE-CALUX assay was less than 15 %. The (anti)estrogenic effect of KHM was found to be not significantly different from that of the background solvent control, whereas KHF exhibited a weak but significant antiestrogenic effect (88 % of that of the solvent control, data not shown).

Ex vivo effect of SPE-HPLC F1 extract of PCB spiked serum

After having demonstrated the *in vitro* effect of the PCB congeners on ER-mediated transactivation in MVLN cells by direct exposure (see "*In vitro* effect of PCBs on ER transactivation: direct exposure of MVLN cells"), we wanted to evaluate the recovery and effect of POPs after reconstitution of F1 serum extracts.

Serum control samples (KHM) were spiked with the three PCBs (138, 153, 180) at low, mean, and maximum concentration levels (Table 2), and the SPE-HPLC F1 extracts were applied to the ERE-CALUX assay. In the absence and presence of E2, the fraction F1 of KHM sera spiked with mean and maximum PCB levels significantly reduced the luciferase expression (Fig. 7a) compared with the respective procedural blank controls (blank ± E2). As observed for the direct *in vitro* exposure (Fig. 5), the F1 spiked serum extract with the mean PCB level (Table 2) decreased the E2-induced luciferase activity to the basal level of the blank solvent control (Fig. 7a).

To evaluate the effect of F1 extract dilution on the ER transactivities, serum control samples (KHM) spiked with
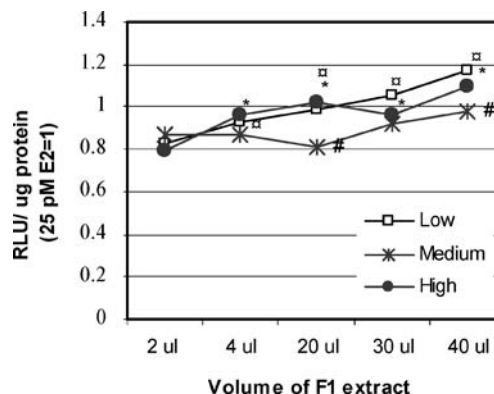

Fig. 6 Effect of F1 serum extracts on ER-mediated transactivation. Dose-response curves of serial different volumes of F1 serum extracts with high, medium, and low content of PCB 153 in the presence of 25 pM E2. The data are related to the dose response of the respective solvent control, which is set to 1. ¤ $p<0.05$ vs. 2 μl (low), * $p<0.05$ vs. 2 μl (high), and # $p<0.05$ vs. 2 μl (medium). RLU per microgram of protein (see legend to Fig. 4)

two different mixtures of the three PCBs, Mix1 and Mix2 (Table 2), were applied to SPE-HPLC. These mixture F1 extracts did not significantly affect the basal ER-mediated luciferase activity (data not shown), but did significantly decrease the E2-EC$_{40}$-induced luciferase activity by 30 % compared with the solvent E2 control (Fig. 7b). The antiestrogenic effect of Mix1 was counteracted by dilution of the sample by 10 times, whereas the inhibiting effect of Mix2 could not be abolished by a dilution factor of 10.

Ex vivo effect of SPE-HPLC F1 extract of selected test serum samples

Inuit serum was extracted and analyzed for xenoestrogenic activity as undiluted and upon 5 and 10 times dilution. Some of the serum extracts decreased the E2-induced luciferase activity, which was counteracted upon dilution (Fig. 8a), as was observed for the PCB spiked serum extracts (Fig. 7b). It is noteworthy that for some samples the 10 times dilution did not abolish the antiestrogenic F1 effect (Fig. 8a, samples 3 and 4), which can be compared with the spiked Mix2 data (Fig. 7b).

Furthermore, we tested whether titration with E2 at different concentrations could compete with the antiestrogenic effect of POPs in Inuit serum extracts (Fig. 8b). In the presence of 25 pM (E2-EC$_{40}$), all the extracts tested significantly decreased the E2-induced transcriptional activation to 47–72 % of the solvent E2–25 pM control. Although titration with an E2 concentration of 150 pM (E2max) did enhance the E2-mediated reporter activity, the extracts still inhibited the ER activity by 20–30 % relative to the E2-150 pM control. However, at twice E2max (300 pM) for 50 % of the samples, the inhibiting effects were counteracted (Fig. 8b).
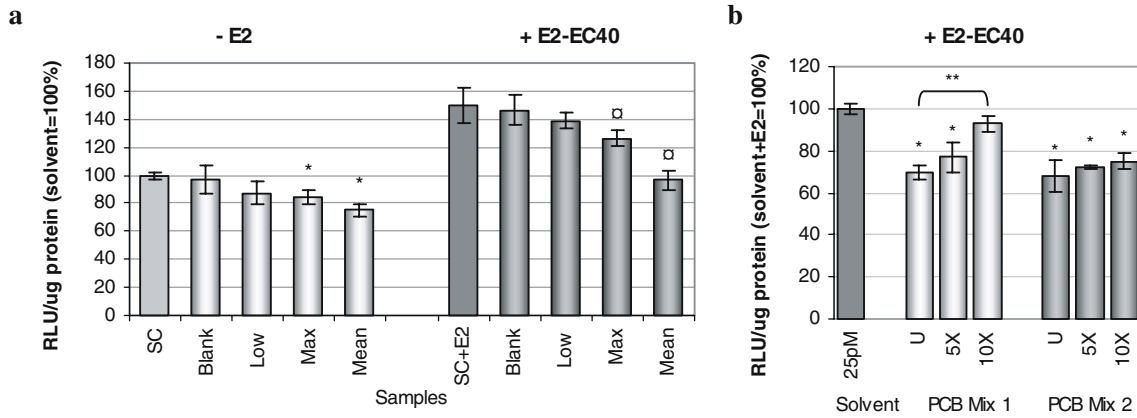
Fig. 7 Effects of PCB spiked serum F1 extract on ER-mediated transactivation. **a** Male serum control (*KHM*) was spiked with a mixture of PCB 138, PCB 153, and PCB 180 at low, maximum, and mean concentration levels, respectively; mimicking the concentration measured in Inuit serum samples (see Table 2). As a procedural blank control an identical KHM sample (without any further spiking) was extracted and analyzed in parallel. Solvent control (*SC*) 100 %, * $p < 0.05$ vs. blank, ¤ $p < 0.05$ vs. blank (+E2). **b** MVLN cells were cotreated with 25 nM E2 and the reconstituted F1 extract of serum was spiked with mixtures of the three PCB congeners: *Mix1* (PCB 138 3.3 μg/l, PCB 153 4.8 μg/l, and PCB 180 4.9 μg/l) and *Mix2*, which is twice the concentration. The RLU per microgram of protein is expressed relative to 25 pM E2=EC$_{40}$. *U* undiluted, *5×* and *10×* 5 and 10 times dilution. * $p < 0.05$ from 25 pM E2, ** $p < 0.05$ from undiluted sample. RLU per microgram of protein (see legend to Fig. 4)

Comparison of (anti)estrogenicity in serum SPE-HPLC extracts from two different laboratories

To compare interlaboratory SPE-HPLC results, the following analysis was carried out. Serum samples previously fractionated by SPE-HPLC by our colleagues in another laboratory (Institute of Public Health, University of Southern Denmark; for the method see [23]) and analyzed in our laboratory for ERE-CALUX activity were extracted again in our newly established SPE-HPLC system followed by

Fig. 8 ERE-CALUX activity of F1 serum extracts upon dilution and titration with E2. **a** Inuit serum extracts (*1* female, *2–6* male) were diluted before activity measurements, and were analyzed undiluted (*U*), or with 5 times dilution (*5×*) and 10 times dilution (*10×*). MVLN cells were coexposed to the F1 serum extracts and 25 pM E2. **b** E2 titration of female Inuit F1 serum extract. MVLN cells were coexposed to the F1 extract and 25 pM E2, 150 pM E2 or 300 pM E2. 25pM E2=EC$_{40}$, E2-150 pM=EC$_{100}$ (E2 max), E2-300 pM=2×EC$_{100}$ (2×E2max). * Significantly different from 25 pM E2. ** Significantly different ($p < 0.05$) from 150 pM E2. The samples in **a** and **b** are not identical. RLU per microgram of protein (see legend to Fig. 4)
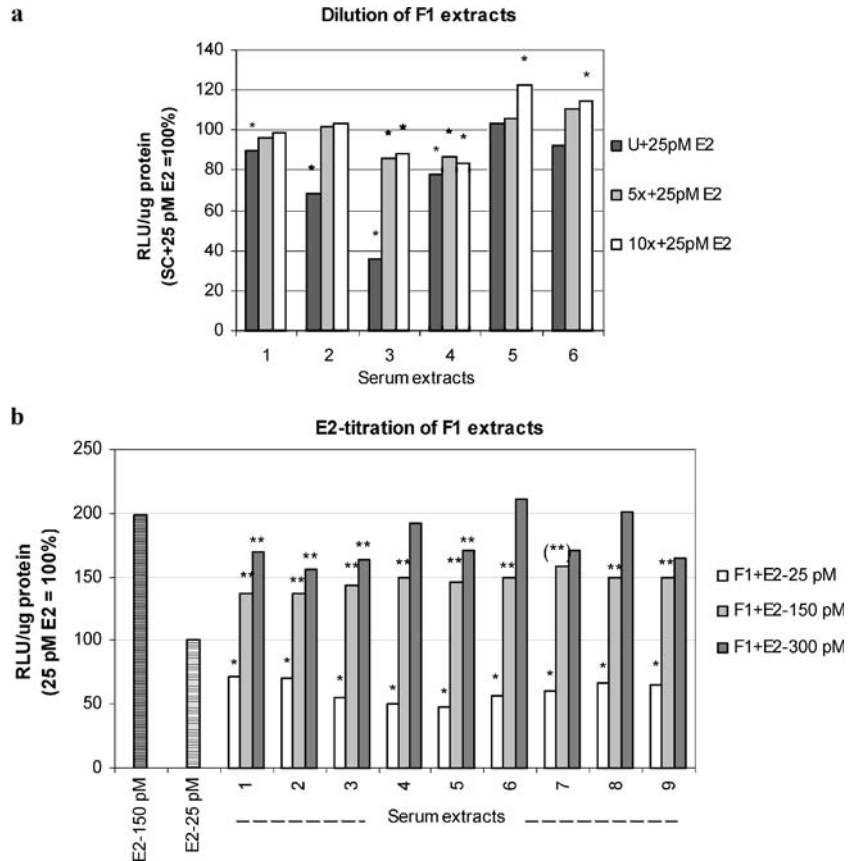
ERE-CALUX activity measurement in the absence and presence of 25 pM E2 (Table 5). The (anti)estrogenicity of each extract tested was evaluated by significance comparison with their corresponding solvent controls. High interlaboratory correlation was found; the effects of all previous and retested serum extracts showed the same effect on ER activity, where 60 % of the comparison was within the expected variance of triple determinations.

Discussion

To assess *in vitro* and *ex vivo* the (anti)estrogenic activity of the sum of accumulated POPs in the human serum extracts we established, validated, and documented the setup of an SPE-HPLC serum extraction method in combination with an ERE-CALUX reporter gene assay [31, 33]. The SPE-HPLC method used was similar to that in previous reports [23, 26]. SPE-HPLC extraction followed by GC-MS analysis, recovery, and/or ERE-CALUX analysis verified that the serum fractions F1 and F2 (F2.1 and F2.2) contained the lipophilic POPs and endogenous sex hormones, respectively.

Additionally, our results validated the reconstitution of the serum extracts and the sensitivity of the bioassay to the estrogenic compounds, e.g., the median estrogenic response measured in F2.2 was 8 times higher in female than in male extracts. Considering the endogenous E2 levels (males 0–130 pM and females 210–1000 pM; PerkinElmer Delfia kit 1244-056), these responses corresponded approximately to what could be expected. Furthermore, the maximal effect of E2 (150 pM), being sixfold more than that of the solvent control, was further increased upon exposure to F2.1 and F2.2 to sevenfold and ninefold, respectively, supporting the use of this system for assessment of the interactions of compounds in the sample. Therefore, we suggest that the activity determined by ERE-

CALUX upon exposure of the MVLN cells to the F1 extract reflects the agonistic/antagonistic and additive/synergistic interactions of the xenoestrogenic compounds in the actual serum sample that can interfere with the ER functions without the interference of endogenous hormones.

Recovery of POPs after reconstitution of the SPE-HPLC F1 extract of PCB spiked (PCBs 138, 153 and 180) serum control samples, and ERE-CALUX activities of F1 compared with the effects observed upon direct cell exposure to the chemicals were in good agreement. These PCBs have been reported to possess antiestrogenic activities [16, 35], and this is in accordance with our findings both by *in vitro* direct exposure and by *ex vivo* analysis after SPE-HPLC cleanup of the spiked serum. Also the ER antagonizing effect of the F1 extract of serum spiked with the three PCBs in a mixture was similar to that in the direct exposure experiment. Interestingly, the ER repression found by the PCBs in a mixture upon direct as well as by F1 extract exposure could not be counteracted by 10 times dilution, which revealed the presence of the PCBs in the reconstituted extract after SPE-HPLC extraction. However, for the actual serum F1 extract dilutions, some samples abolished the inhibiting effect and the ER activity reached that of the procedural blank controls, whereas for other sample extracts the dilution did not bring the activity to the blank control level, which might be due to higher content and/or different profiles of POPs in these serum samples.

Similarly, titration by increasing E2 concentrations could to some extent reverse the ER antagonistic effect of the actual POPs in serum F1 extracts; while the E2-EC_{100} could not fully counteract the antagonistic effect of F1, raising the E2 concentration to twice the maximum E2 effective concentration (EC_{200}) abolished the antagonizing effect of 50 % of the F1 extracts, suggesting a concentration/affinity-dependent competition between E2 and xeno-hormones for ER.

Table 5 Comparison of estrogen receptor (*ER*) transactivities in serum SPE-HPLC F1 extracts from two different laboratories

Sample	SPE-HPLC, IPH-SDU		HPLC, IPH (CMT)-AU	
	−25 pM E2	+25 pM E2	−25 pM E2	+25 pM E2
1	ND	↓(48)	→	↓(10)
2	→	↓(26)	→	↓(10)
3	↓(20)	↓(31)	↓(22)	↓(37)
4	↓(50)	↓(25)	↓(33)	↓(43)
5	→	↓(29)	→	↓(16)
6	ND	↓(40)	→	↓(20)
7	ND	↓(34)	↑(43)	↓(10)
8	ND	↓(34)	↑(26)	→[a]

The serum samples were of Greenlandic origin. The numbers in *parentheses* give the decreased or increased value compared with the solvent control, in percent. All of the comparisons showed the same effect on ER activity; 60 % of the comparisons were within the expected analyses variance of triple determinations

IPH-SDU Institute of Public Health, University of Southern Denmark, *IPH (CMT)-AU* Institute of Public Health, Unit of Cellular & Molecular Toxicology, University of Aarhus, ±25 pM 17β-estradiol (*E2*) ERE-CALUX activity was determined in the absence or presence of 25 pM E2 (EC_{40}); *ND* not determined, → ERE-CALUX activity not different from that of the solvent control, ↓ ERE-CALUX activity significantly ($p < 0.05$) decreased compared with the solvent control, ↑ ERE-CALUX activity significantly ($p < 0.05$) increased compared with the solvent control

[a] The only difference observed between the two laboratories

By analyzing serum samples with different PCB 153 levels, we showed that the system is refined to detect the xenoestrogenic compounds at lower, medium, and high levels of POPs, and that the ER responses of F1 were in a linear range.

Our results confirmed and validated the SPE-HPLC extraction procedure for application in the ERE-CALUX bioassay for detection of actual xenoestrogenic serum activities. In support, previous comparisons of ERE-CALUX assay (MVLN) to E-screen [27, 36] showed a comparable sensitivity for E2 and a good correlation between the estrogenic potencies of other compounds (R=0.9891). Furthermore, comparisons of our present SPE-HPLC-ERE-CALUX data with data obtained from serum SPE-HPLC extractions performed in another laboratory [23] showed good agreement, indicating a good inter-laboratory correlation.

Conclusion

To our knowledge the combination of SPE-HPLC purification with ER transactivation assay to assess the combined xenoestrogenic effect of lipophilic POPs in human serum has not previously been reported. We have thoroughly evaluated this system, in which ER additive/synergistic and agonistic/antagonistic effects are integrated giving the net bioactivity. The complex nature of exposure patterns and accumulation levels of known and unknown compounds necessitates assessment tools other than the pure chemical analysis in which single compounds are detected individually. The additive/synergistic and/or antagonistic effects of the large number of compounds present in human serum cannot be predicted on the basis of individual compounds considering their mutual interactions. The present study demonstrates how to assess the combined net effect of lipophilic POPs in human serum giving an overall image of their potential biological xenoestrogenic activity.

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