

# Effect-directed analysis of endocrine-disrupting compounds in multi-contaminated sediment: identification of novel ligands of estrogen and pregnane X receptors

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**Abstract** Effect-directed analysis (EDA)-based strategies have been increasingly used in order to identify the causative link between adverse (eco-)toxic effects and chemical contaminants. In this study, we report the development and use of an EDA approach to identify endocrine-disrupting chemicals (EDCs) in a multi-contaminated river sediment. The battery of in vitro reporter cell-based bioassays, measuring estrogenic, (anti)androgenic, dioxin-like, and pregnane X receptor (PXR)-like activities, revealed multi-contamination profiles. To isolate active compounds of a wide polarity range, we established a multi-step fractionation procedure combining: (1) a primary fractionation step using normal phase-based solid-phase extraction (SPE), validated with a mixture of 12 non-polar to polar standard EDCs; (2) a secondary fractionation using reversed-phase-based high-performance liquid chromatography (RP-HPLC) calibrated with 33 standard EDCs; and (3) a purification step using a recombinant estrogen receptor (ER) affinity column. In vitro SPE and HPLC profiles revealed that ER and PXR activities were mainly due

to polar to mid-polar compounds, while dioxin-like and anti-androgenic activities were in the less polar fractions. The overall procedure allowed final isolation and identification of new environmental PXR (e.g., di-iso-octylphthalate) and ER (e.g., 2,4-di-*tert*-butylphenol and 2,6-di-*tert*-butyl- -methoxy-*p*-cresol) ligands by using gas chromatography coupled with mass spectrometry with full-scan mode acquisition in mid-polar fractions. In vitro biological activity of these chemicals was further confirmed using commercial standards, with di-iso-octylphthalate identified for the first time as a potent hPXR environmental agonist.

**Keywords** EDCs · Emerging pollutants · Multi-contaminated sediment · Multi-step fractionation

## Introduction

Aquatic systems are the receptacle for natural and anthropogenic chemical contaminants, including endocrine-disrupting chemicals (EDCs), that may trigger adverse effects on wildlife and humans [1]. With respect to the implementation of the European Water Framework Directive, which demands a good ecological status by 2015, an increasing expectation to analyze and monitor hazardous chemicals has emerged during the last decade [2]. However, environmental monitoring based on chemical analysis of priority pollutants often fails to identify effective chemicals due to the great diversity of environmentally occurring contaminants which impedes accurate chemical risk assessment. Hence, development of novel strategies for the identification of pollutants that indeed trigger adverse effects could tackle these challenges.

Effect-directed analysis (EDA) aims at identifying biologically active chemicals by using fractionation procedures that reduce the complexity of environmental matrices and allow the progressive isolation of chemicals detected by bioassays

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[3]. In EDA assessment of EDC-mediated activities, the fractionation procedure often consists of a multiple-step approach using, for instance, a combination of solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) [4, 5]. However, the majority of existing fractionation methods for sediment extract was mainly developed to study a single class of compounds (i.e., non-polar or polar compounds [3, 6]) and only few studies have dealt with multiple biological endpoints [7, 8]. One common feature of these few studies is the co-occurrence of several biological activities in the same final fractions or group of fractions. Hence, there is still a need to improve and validate fractionation strategies allowing both (1) the separation of chemicals exerting a wide range of polarities (i.e., polar, semi-polar, and non-polar chemicals) and (2) the isolation of active compounds in the final fractions.

To date, EDA has been successfully used for the identification of EDCs able to interfere with hormonal and xenobiotic receptors, such as estrogenic [6, 9], androgenic [4], anti-androgenic [4, 10], and dioxin-like chemicals [11]. Over and above these EDC families, many environmental chemicals can bind to other nuclear receptors that play a critical role in the regulation of the endocrine system and metabolism. For instance, recent studies have shown the occurrence of human pregnane X receptor (hPXR) ligands in river sediment, surface water river, and STP effluents [12–14]. This receptor is known to be activated by a wide range of environmental pollutants including pharmaceuticals and personal care products [12, 15–17] and has been proposed as a xenosensor for the detection of a broad range of environmental chemicals presenting different physico-chemical properties (e.g., polarity and solubility). In addition, because hPXR plays a crucial role in the xenobiotic detoxification and the clearance of endogenous hormones [18], abnormal modulation of its activity could lead to an

adverse effect. Therefore, the identification of environmental pollutants that can activate this receptor may enhance knowledge on environmental risk.

By using a battery of complementary *in vitro* bioassays, we previously reported the occurrence of complex toxicity profiles, including PXR-like activity, in a multi-impacted French river [13] where ecotoxicological impacts on fish were evidenced [19]. We showed that bioanalytical characterization based on a mass balance analysis derived from targeted chemical analysis provided only partial information on the identity of chemicals detected by the bioassays. The aim of the present study was thus to implement an EDA approach based on multi-steps fractionation in order to isolate and identify active chemicals occurring in this sediment. This protocol, which has been validated using a broad range of standards, uses normal phase SPE as a first fractionation step, followed by a second fractionation step using reversed-phase-based high-performance liquid chromatography (RP-HPLC). Finally, we focused on the chemical identification of hPXR and human estrogen receptor (hER) ligands. For this latter purpose, an affinity column based on immobilized recombinant hER $\alpha$  was used to purify estrogen receptor (ER) ligands in active fractions [20].

## Materials and methods

### Chemicals and reagents

Chemicals used as standards for analytical methods (Table 1) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France), LGC standard (Molsheim, France), and Cluzeau (Sainte Foy La Grande, France). Acetonitrile (ACN), dichloromethane (DCM), isooctane, and methanol (MeOH; HPLC reagent grade, Scharlau, Spain) were purchased from

**Table 1** Chemicals and analytical methods used in this study

Classes (providers)	Compounds	Analytical method (reference)
PAHs (LGC standard)	Naphtalene, acenaphthylene, acenaphthene, fluorene, anthracene, fluoranthene, dibenzothiophene, pyrene, benzo[a]anthracene, triphenylen, chrysene, benzo[b/j/f]fluoranthene (Bb/j/kF), benzo[e]pyrene, benzo[a]pyrene, indenopyrene, dibenzo[a,h]anthracene, benzoperlylen, and triphenyl phosphate	GC-MS (SIM) [22]
PCBs (LGC standard)	PCB08/18/29/50+28/52/44/104/66/101/87/154+77/188/153/105/138/126/187/128/200/180/170/195/206/209	GC- $\mu$ ECD [22]
BDEs (LGC standard)	BDE47/119/99/153	GC- $\mu$ ECD [42]
OCPs (Cluzeau)	Hexachloro-benzene, lindane, heptachlor, heptachlore epoxide, 2,4'DDE, <i>cis</i> -chlordane, <i>trans</i> -nonachlore, 4,4'DDE, dieldrin, 2,4'DDD, 4,4'DDD, 2,4'DDT, 4,4'DDT, and mirex	GC- $\mu$ ECD [42]
Steroids (Sigma)	Estriol, norethindrone, testosterone, ethnyloestradiol, estrone, 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol, levonorgestrel, progesterone, and mestranol	UPLC-MS/MS [43]
Pharmaceuticals (Sigma)	Caffeine, carbamazepine, diazepam, nordiazepam, amitriptyline, doxepine, imipramine, ibuprofene, paracetamol, ketoprofene, naproxene, aspirine, diclofenac, gemfibrozil, clenbuterol, salbutamol, terbutaline, theophylline, alprazolam, bromazepam, fluoxetine, clotrimazole, and fenofibrate	RRLC-MS/MS [44]
Alkylphenols (Cluzeau)	NP2EO, NP1EO, NP1EC, 4-nonylphenol, bisphenol A, and 4-tert-octylphenol	UPLC-MS/MS [45]

ICS (Belin Beliet, France). Luciferin and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France).

### Study site and sample preparation

The study site is located on the Réveillon River, a small French stream subjected to mixed anthropogenic pressure (i.e., agricultural and urban; GPS coordinates: N 48°34'00"; E 2°32'09"). Sediment was sampled in April 2009 with a grab, sieved (mesh size, 1 mm), freeze-dried, homogenized, and stored in amber glass bottles at  $-20^{\circ}\text{C}$  before analysis.

Sediment was extracted using an accelerated solvent extraction systems (ASE® 350, Dionex, France) equipped with a 10-ml stainless extraction cell vessel. Five grams of freeze-dried sediment were placed in an extraction cell and thoroughly mixed with sand in order to fill the volume of the extraction cell. A mixture of MeOH/DCM (50:50, v/v) was used for the extraction. The operating conditions were the following: extraction temperature,  $75^{\circ}\text{C}$ ; extraction pressure, 1,500 PSI; preheating period, 5 min; static extraction, 5 min; number of extraction cycles, three; final extraction volume, 25 ml; flush volume, 60 % of the cell volume; and nitrogen purge, 60 s. These extraction conditions were previously determined as suitable for the extraction of a broad range of chemicals in

terms of both physico-chemical properties and biological activities [21]. The extracts were then evaporated to residues using an EZ-2® solvent evaporator system (Genevac, UK) and dissolved in MeOH/DCM for storage. For in vitro bioassays, aliquots of MeOH/DCM extract were dried to a few microliters and dissolved in DMSO. For fractionation experiments, sediment extracts were dissolved in heptane.

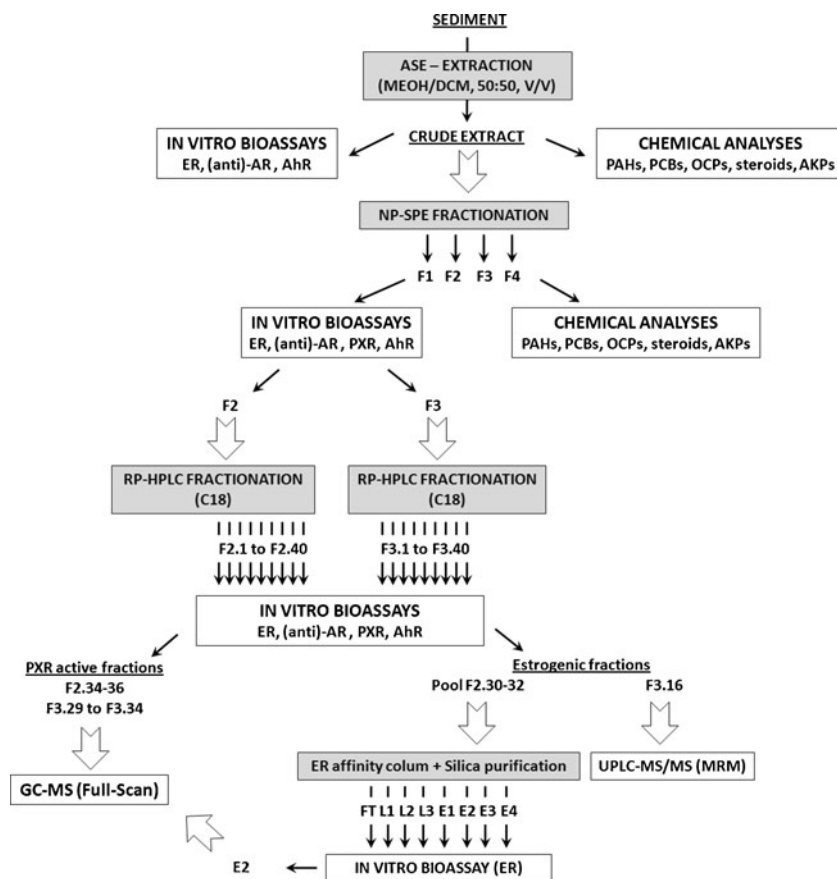
### Fractionation strategy

Fractionation of sediment extracts were performed using a sequential procedure based on normal phase-SPE (NP-SPE), RP-HPLC, and recombinant ER-based affinity column (Fig. 1). NP-SPE and RP-HPLC fractionations have been calibrated for the isolation of chemicals exerting a broad range of polarity. This calibration is detailed in the results and discussion section.

### Polarity-based fractionation

NP-SPE fractionation was assessed on sediment extract dissolved in 1 ml of heptane, using silica cartridges (500 mg–6 cm<sup>3</sup>; Supelco, Saint-Quentin-Fallavier, France) previously conditioned with 5 ml of heptane. Sequential elution was performed using heptane (10 ml), heptane/DCM (50:50, v/v;

**Fig. 1** Overall fractionation strategy used for the isolation and identification of PXR and ER ligands in sediment extract. (FT flow-through; washing fractions, L1–L3; elution fractions, E1–E4)



10 ml), ethyl-acetate (10 ml), and MeOH/H<sub>2</sub>O (50:50, v/v; 10 ml) leading to four fractions (F1 to F4) of increasing polarity. The obtained fractions were then evaporated to dryness under nitrogen flow (F1, F2, and F3) or using the EZ-2 evaporator (F4) and then dissolved in 1.5 ml ACN. Additionally, the influence of a purification step using an alumina-silica column on the biological activity of F1–F4 fractions was also assessed. This step allows the elimination of polar to semi-polar compounds and is dedicated to the analysis of non-polar chemicals, such as polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), and organochlorinated pesticides (OCPs) [22].

The second fractionation step was performed using an RP-HPLC system equipped with a C18 column (Poursuit C18, 5 µm, 250×4.6 I.D.; Agilent Technologies, Les Ulis, France), by using an H<sub>2</sub>O/ACN gradient at a starting ratio of 80:20 (v/v). The run was performed at 25 °C at a flow rate of 1 ml/min with the following gradient program: 0–10 (80:20), 60 (55:45), 100–120 (0:100), and 120.01–125 min (80:20). In the present study, F2 and F3 fractions were fractionated, leading to 40 fractions of 3 ml each (F2.1 to F2.40 and F3.1 to F3.40). The collected fractions were evaporated to dryness using the EZ-2 solvent evaporator system and then dissolved in 200 µl of ACN for storage. Aliquots of this ACN extract were then transferred to DMSO for biological analysis.

#### *ER binding affinity-based fractionation*

An hER $\alpha$  affinity column was used to selectively isolate human ER $\alpha$  ligands in HPLC fractions exerting estrogenic activity (Fig. 1), as previously described [20]. All the experiments were carried out at 4 °C. The recombinant histidine-hER $\alpha$  was first immobilized on an Ni-NTA agarose phase (Quiagen, France) in a polytetrafluoroethylene column. In order to eliminate impurities, immobilized receptors were then washed three times with 500 µl of washing buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 % glycerol, 0.1 mg/ml BSA, and 10 mM imidazole). Then, the test sample was diluted (1/100) in washing buffer and loaded on the column. After three washes, hER $\alpha$  ligands were eluted in four fractions (E1 to E4) using an elution buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1 mg/ml, BSA, and 200 mM imidazole), and the remaining material was eluted using ethanol. Fractions corresponding to flow-through (FT) and washes (L1 to L3) were also collected and processed for further (bio)analysis. The resulting fractions were then concentrated and purified in order to suppress any potential matrix effect in the in vitro bioassays due to buffer components. For this purpose, fractions were first concentrated to 10 µl, redissolved in heptane and then purified on silica cartridges using the NP-SPE fractionation procedure as described above.

#### *Biological analysis and bio-TEQ calculation*

Procedures for routine cell culture and sample in vitro testing have been previously described [12]. In brief, MELN [23], MDA-kb2 [24], HG5LN-HPXR [16], and PLHC-1 [25] cell lines were used to assess estrogenic, (anti)-androgenic, PXR-like, and dioxin-like activities, respectively. For the luciferase assay in MELN, MDA-kb2, and HG5LN-hPXR bioassays, cells were seeded in opaque 96-well plates at 10<sup>5</sup> cells/well. After 24 h, the cells were exposed in triplicate to a serial dilution of crude extract or fractions and incubated for 16 h. The solvent (DMSO) final concentration was always 0.5 % (v/v) in the culture medium. Luciferase activity was assessed by addition of D-luciferin (0.3 mM) and measurement on a microtiter plate luminometer (MicroBeta, Wallac, Perkin-Elmer). For the EROD assay, PLHC-1 cells were seeded in transparent 96-well plates at 5.10<sup>4</sup> cells/well. After 24 h, the cells were exposed to test samples for 4 and 24 h in order to distinguish between persistent and readily metabolized chemicals. After the addition of 5 µM 7-ethoxyresorufin, EROD activity was assessed on a spectrofluorimeter at excitation/emission wavelengths of 400/460 nm (Sapphire2, Tecan, Lyon, France).

Dose-response curves from bioassays were modeled using the Microsoft Excel™ macro RegTox 7.0.3 (freely available at [http://www.normalesup.org/~vindimian/fr\\_index.html](http://www.normalesup.org/~vindimian/fr_index.html)). This was used to calculate biological toxic equivalents (bio-TEQ), which were defined as the ratio of the EC<sub>20</sub> of the reference compounds to those of the sample extracts in a given assay, as detailed previously [13].

#### *Chemical analyses and chem-TEQ calculation*

##### *Targeted chemical analyses in sediment and mass balance analysis*

Chemical analyses of steroids, alkylphenols, bisphenol A (BPA), PAHs, PCBs, and OCPs are listed in Table 1. Chemical toxic equivalents (chem-TEQ) were then determined according to the following equation: chem-TEQ =  $\sum C_i \times \text{TEF}_i$ , where  $C_i$  is the concentration of compound  $i$  in the extract or fraction and  $\text{TEF}_i$  is the toxic equivalent factor (TEF) defined as the ratio of the EC<sub>20</sub> of the reference compound to the EC<sub>20</sub> of compound  $i$ . All TEF values used in this study are provided in Table S1 (Electronic supplementary material (ESM)). They have been determined in each individual assay by testing a range of concentrations (up to 10 µM) of individual chemicals.

##### *Chemical identification of unknown compounds isolated in RP-HPLC fractions*

Gas chromatography coupled with mass spectrometry (GC-MS) was used to identify the unknown compounds in active

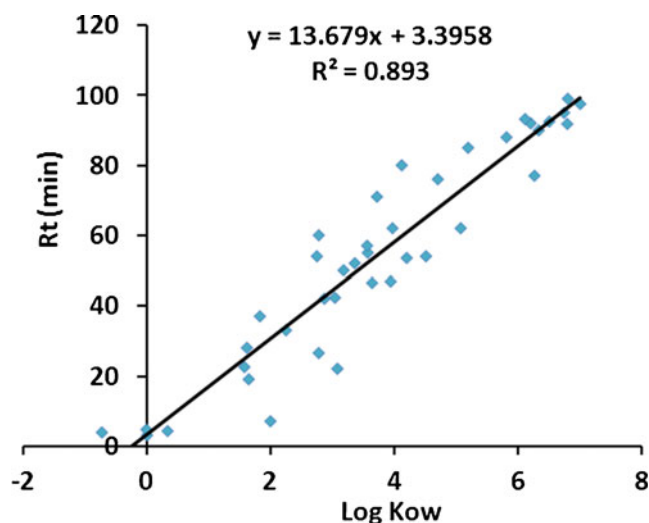
fractions. Analyses were assessed using a GC (Agilent 6890, France) equipped with a HP-5MS fused silica capillary column, coupled to a MS (Agilent 5973) and an autosampler-injector (Agilent 7683). Helium was used as carrier gas at a constant flow rate of 1.3 ml/min. Sample aliquots of 1  $\mu$ l were injected in splitless mode at an injector temperature of 250 °C. The oven temperature was increased from 50 to 300 °C at a rate of 4 °C/min and held for 6.5 min. The mass range scanned was 50–500  $m/z$  using electron impact ionization at 70 eV.

## Results and discussion

### Calibration of the NP-SPE and RP-HPLC fractionations

NP-SPE fractionation procedure has been first calibrated for the isolation of organic compounds by using a mixture of 12 active EDCs (E1, E3, 4-tert-octylphenol, BPA, 2,4'DDT, fenofibrate, triphenyl phosphate, clotrimazole, dibenzo[a,h]anthracen, benzo[k]fluranthen, PCB126, and TCDD), which covered a large range of hydrophobicity (i.e.,  $\log K_{OW}$  comprised between 2 and 7). Several different cartridges (silica, alumina, Oasis HLB, C18) were first tested for the isolation of active chemicals (data not shown), and silica cartridges were identified as allowing the best recoveries (Table 2). The established calibration procedure showed a weak matrix effect and good extraction recoveries (i.e., higher than 70 %) for the tested chemicals, as well as an overall weak matrix effect, except for clotrimazole with 63 % of recovery in the spiked blank sediment extract (Table 2).

The elution gradient used for RP-HPLC fractionation was calibrated using a set of 33 chemicals belonging to different organic contaminant families and exerting a broad range of hydrophobicity ( $\log K_{OW}$  between 0 and 7) (Table S1 in the ESM). The retention time of standard chemicals was correlated to their  $\log K_{OW}$  ( $R^2=0.893$ ) (Fig. 2). The RP-HPLC elution profile of these standard EDCs after an NP-SPE pre-fractionation step was then established and is reported in Table 3. These data helped to validate the established



**Fig. 2** RP-HPLC calibration with 33 standard chemicals: correlation between  $\log K_{OW}$  and retention time ( $Rt$ )

fractionation method for the separation of a wide range of EDCs, prior to its application to environmental samples.

### Biological and chemical analyses of sediment crude extracts

Estrogenic, anti-androgenic, PXR- and dioxin-like activities were detected in the crude extracts of the Réveillon sediment. Dose response curves allowed bio-TEQ values to be established for each of these activities (Fig. 3). Overall, the bio-TEQ values were quite similar to those previously reported at the same site in sediment sampled in 2005 (data in Fig. 3), suggesting that this river is subject to a constantly high contamination level.

The targeted chemical analyses revealed the presence of trace levels of natural but not synthetic hormones (E2, testosterone, and progesterone), as well as detectable levels of miscellaneous organic contaminants, such as OCPs, PAHs, PCBs, PBDEs, alkyphenols, and BPA. However, based on a mass balance calculation, the chemical analyses explained only a small part of the activities detected using bioassays (Table S2 in the ESM). These observations are overall in line with those previously reported for the same

**Table 2** Recoveries of the primary fractionation procedure on silica cartridges (in percent, means  $\pm$  SD,  $n=3$ )

No compound was detected in F4  
*4tOP* 4-tert-octylphenol, *DaA* dibenzo[a,h]anthracene, *TPP* triphenyl-phosphate, *E1* estrone, *17 $\beta$ -E2* 17 $\beta$ -estradiol, *BPA* bisphenol A

	F1 (heptane)	F2 (heptane/DCM)	F3 (EA)
Spiked solvent	2,4'DDT	99 $\pm$ 1	4tOP 91 $\pm$ 7
	DaA	98 $\pm$ 4	Fenofibrate 98 $\pm$ 8
	BkF	99 $\pm$ 2	TPP 94 $\pm$ 9
	PCB126	98 $\pm$ 4	
Spiked blank sediment extract	2,4'DDT	76 $\pm$ 13	4tOP 83 $\pm$ 15
	DaA	83 $\pm$ 17	Fenofibrate 82 $\pm$ 9
	BkF	74 $\pm$ 10	TPP 88 $\pm$ 16
	PCB126	80 $\pm$ 15	
			E1 88 $\pm$ 17
			17 $\beta$ -E2 108 $\pm$ 14
			BPA 97 $\pm$ 5
			Clotrimazole 118 $\pm$ 6
			E1 87 $\pm$ 20
			17 $\beta$ -E2 99 $\pm$ 19
			BPA 85 $\pm$ 5
			Clotrimazole 63 $\pm$ 15

**Table 3** Elution profile of the 30 analytical standards in NP-SPE and subsequent RP-HPLC

	Fractions (min)	SPE fractions		
		F1	F2	F3
HPLC fractions	F1–F8 (1–24)			
	F9 (25–27)			Benzophenone 2
	F10 (28–30)			Prednisolone
	F11 (31–33)			Carbamazepin
	F12 (34–36)			
	F13 (37–39)			Dexamethasone
	F14 (40–42)			Isoproturon
	F15 (43–45)			<i>n</i> -propylparaben
	F16 (46–48)			BPA, $\beta$ E2, and $\alpha$ -zearalanol
	F17 (49–51)			BP1
	F18 (52–54)			EE2 and <i>n</i> -butylparaben
	F19 (55–57)			<i>n</i> -benzylparaben
	F20 (58–60)			Spironolactone
	F21 (61–63)			Ibuprofen and DES
	F22–23 (64–69)			
	F24 (70–72)			BP3 and propiconazole
	F25 (73–75)			
	F26 (76–78)			TPP and clotrimazole
	F27 (79–81)			4tOP
	F28 (82–84)			
	F29 (85–87)			Fenofibrate
	F30 (88–90)	Chrysen		Cypermethryn
	F31 (91–93)	2,4'DDT		Fenvalerate
	F32 (94–96)	Bkf and DaA		
	F33 (97–99)	PCB 126 and TCDD		
	F34–40 (100–120)			

NP nonylphenol, TPP triphenyl-phosphate, BPA bisphenol A, 4tOP 4-tert-octylphenol

site [13]. Therefore, fractionation of the crude extract was implemented in order to isolate and identify the active chemicals responsible for the biological activities.

#### Biological and chemical analyses of NP-SPE fractions

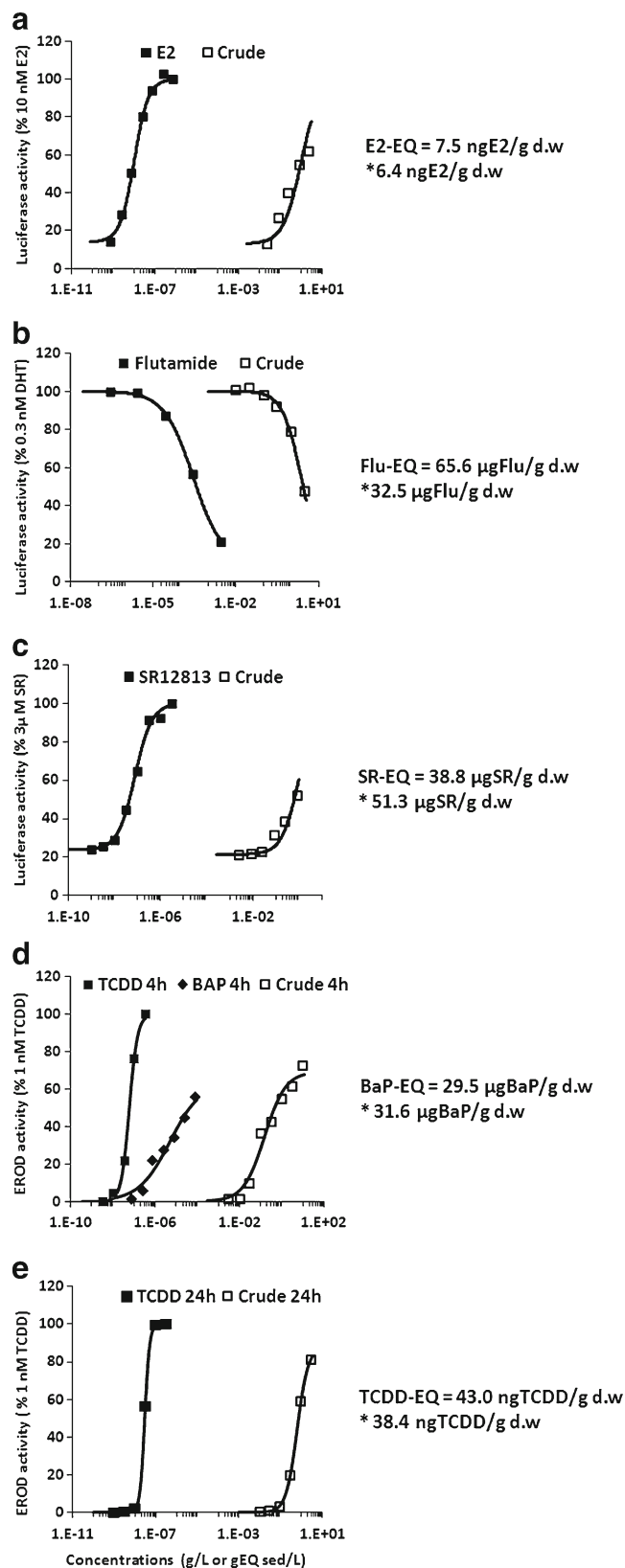
A primary fractionation step was performed in order to obtain an initial characterization of the physico-chemical properties of active ligands. Figure 4 shows that *in vitro* activities were differently distributed between the four fractions, with anti-androgenic and dioxin-like activities mainly detected in F1 and F2, the less polar fractions, while ER and PXR ligands were essentially in mid-polar to polar fractions (F2 and F3).

Overall, comparison of biological activities between crude and F1–F4 pool extracts suggested limited loss of active chemicals associated with this SPE procedure (Fig. 4). In addition, for most of the activities, the sum of the individual fractions' activities was equal to those of crude and pool extracts, suggesting an additive effect of

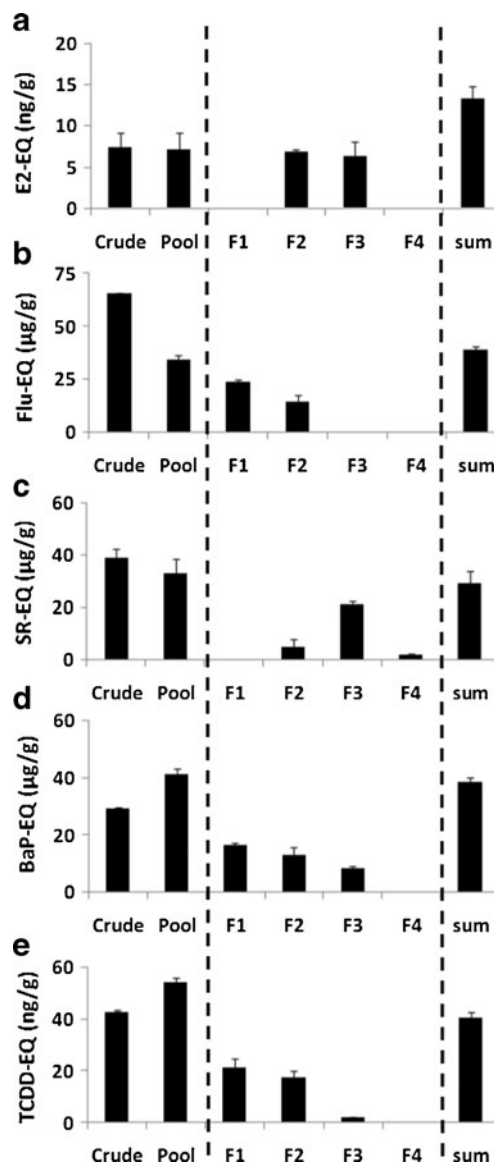
active chemicals. Some exceptions were however noted. A significant difference was noted for anti-androgenic activity, which was higher in the crude extract than in pooled fractions suggesting a loss of anti-androgenic chemicals at this stage. For estrogenic activity, NP-SPE fractionation may have highlighted an inhibitory mixture effect in crude and pooled extracts since the sum of E2-EQ in individual F2 and F3 fractions was higher than that measured in global extracts (Fig. 4). Matrix interference might explain such an anti-estrogenic effect as matrix components are isolated from the active chemicals in the fractionation process.

The purification on an alumina-silica micro-column led to the elimination of polar organic contaminants and

**Fig. 3** Dose–response curves of crude extract of Réveillon sediment for **a** estrogenic, **b** anti-androgenic, **c** PXR-like, **d** BaP-like, and **e** TCDD-like activities. Results are expressed as a percentage of maximal activity induced by **a** 10-nM 17 $\beta$ -estradiol, **b** 0.1-nM DHT, **c** 3- $\mu$ M SR12813, and **d**, **e** 1-nM TCDD. Asterisks, values from Kinani et al. [13] obtained in sediment sampled at the same site in 2005



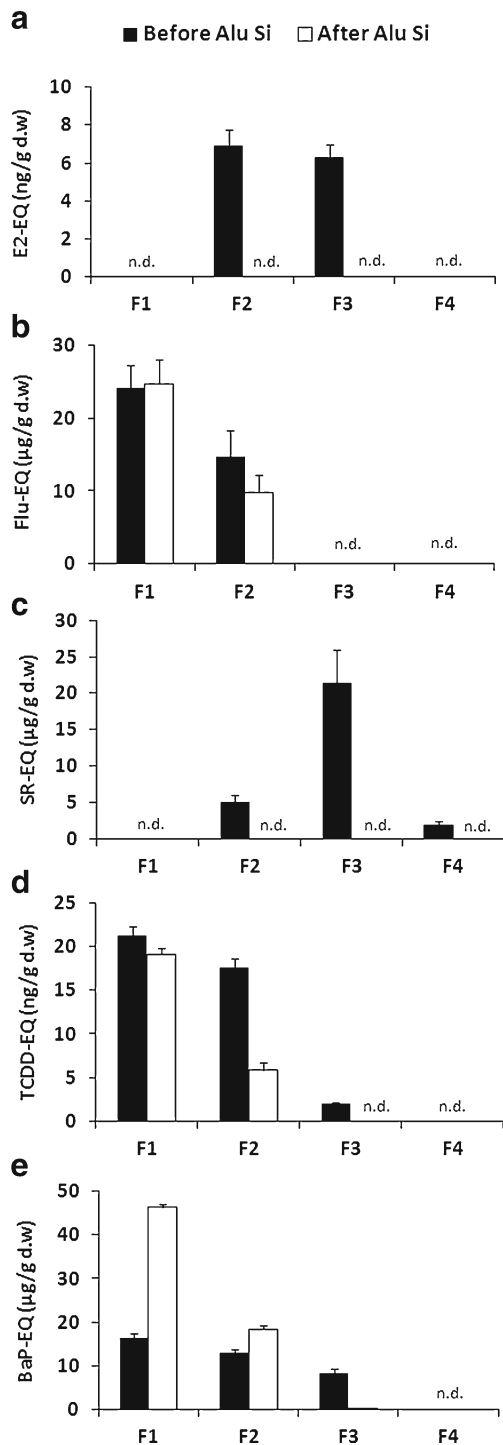
provided further information on bioactive chemicals. This purification step led to the loss of estrogenic and PXR-like activities while anti-androgenic and dioxin-like activities



**Fig. 4** Comparison of **a** estrogenic, **b** anti-androgenic, **c** PXR-like, and **d, e** dioxin-like activities in sediment crude extract and in pooled, individual, and the sum of NP-SPE primary fractions (F1 to F4). Results are expressed as biological toxic equivalents (mean±SD of triplicates)

persisted (Fig. 5). This confirmed that ER and PXR activators were polar compounds whereas AR antagonists and dioxin-like chemicals were mainly non-polar chemicals. Some OCPs, known as anti-androgenic chemicals [10, 26] and AhR active compounds [27], may have contributed to such responses in F1 and F2, respectively. Interestingly, elimination of dioxin-like activity in F3 revealed the occurrence in the sediment extract of polar AhR ligands other than classical dioxin-like chemicals, such as PCBs, PAHs, and PCDD/Fs. These results were in accordance with recent studies reporting the occurrence of such polar AhR ligands in sediment [7, 28, 29].

Finally, targeted chemical analyses on these fractions confirmed the distribution of PAHs, PCBs and OCPs in



**Fig. 5** Comparison of **a** estrogenic, **b** anti-androgenic, **c** PXR-like, and **d**, **e** dioxin-like activities in SPE fractions, before and after their purification on an alumina/silica micro-column. Results are expressed as bio-TEQ (mean±SD of triplicates)

F1, nonylphenol in F2, and BPA and E2 in F3 (data not shown). These chemical patterns closely correlated with the distribution of biological activities although the major contributors remain to be identified, especially in F2 and F3

where estrogenic and PXR-like activities were poorly explained by target chemicals. To this end, RP-HPLC fractionation was performed on these fractions.

#### RP-HPLC fractionation of F2 and F3 primary fractions

Firstly, in order to check potential loss of active compounds at this stage, RP-HPLC fractions were pooled and tested in each bioassay and the resulting activity was compared with that of parent F2 or F3 fractions. As seen in Fig. 6, no alteration of PXR-like, anti-androgenic, and dioxin-like activities could be noted. However estrogenic activity in pooled HPLC fractions was about twofold lower than in the initial F3 and F2 fractions (Fig. 6a). These results suggested a loss of active chemicals associated with the hyper-fractionation procedure. Similar observations have been previously reported in other studies. For instance, Brack et al. [30] noted a strong decrease in EROD activity after fractionation by RP-HPLC whereas calibration of the procedure showed recovery higher than 70 %. These authors attributed this loss to irreversible adsorption of organic matter to the stationary phase of the HPLC column and/or to solvent exchange to a polar solvent such as ACN.

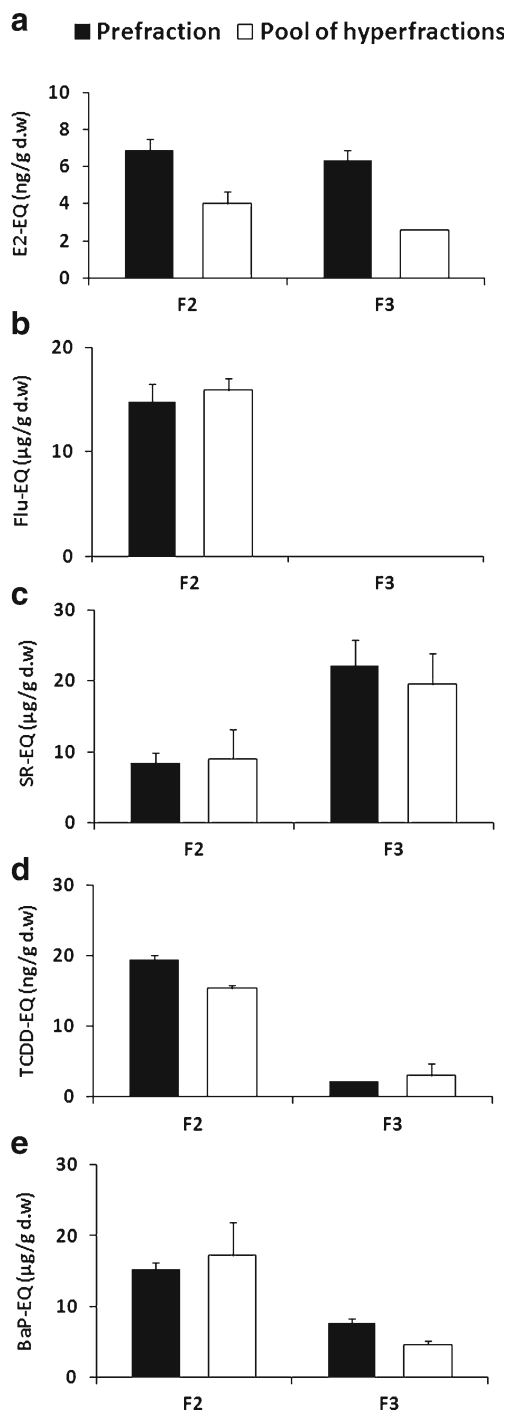
The *in vitro* RP-HPLC profiles of F2 and F3 fractions are presented in Table 4. These profiles highlighted significant dioxin-like activities in two groups of fractions, i.e., F2.24–34 and F3.22–30. This activity was mediated by readily metabolized compounds, such as PAH, since it was almost undetectable after 24 h. Apart from dioxin-like activity, this fractionation step allowed good isolation of ER and PXR activities in a small number of individual fractions. Active fractions for these latter activities were then isolated and further investigated by GC-MS to identify the responsible compounds as described below.

#### Isolation and identification of PXR ligands

In order to identify PXR activators, the fractions F2.34, F2.35, and F2.36 were injected into GC-MS in full-scan mode acquisition. One major peak was detected in F2.35 while no peak was detected in both F2.34 and F2.36. Comparison with the NIST98 mass spectra database allowed identification of di-isooctylphthalate (DIOP) as a potential PXR ligand (Fig. 7).

It is noteworthy that background contamination of samples by laboratory plastics and equipment is a well-known confounding factor when analyzing phthalates in environmental samples. In the present study, since all procedural blanks were found negative for this compound (Fig. 7), we assumed that DIOP was present in the initial sediment extract and did not come from background contamination by experimental procedure.





**Fig. 6** Comparison of activities in individual F2 or F3 SPE fractions (black) and pool of 40 HPLC fractions resulting from each of these primary fractions (white): **a** estrogenic, **b** anti-androgenic, **c** PXR-like, and **d**, **e** dioxin-like activities. Results are expressed as bio-TEQ (mean  $\pm$ SD of triplicates)

Several phthalates have been described as PXR ligands. By using the same cell line as in the present study, Mnif et al. [17] showed that bis(2-ethylhexyl)-phtalate, dibutyl-phtalate, and benzylbutyl-phtalate

were able to activate this receptor. Milnes et al. [31] also showed that diethyl-, benzylbutyl-, bis(2-ethylhexyl)-, dicyclo-hexyl-, dibutyl-, *n*-dipentyl-, *n*-dipropyl-, and di-*n*-hexyl-phtalate were PXR activators in transiently transfected COS-7 cells. To our knowledge, hPXR activation by DIOP is reported here for the first time. To confirm its potency, we exposed HG5LN-hPXR and HG5LN cell lines to a range of concentrations of this chemical. A full dose-response curve was obtained in the HG5LN-hPXR cell line, yielding an SR-EF (SR12813 equivalent factor) value of 0.03. The absence of modulation of luciferase activity in HG5LN cells that does not express hPXR (Fig. 8), confirmed DIOP as a specific activator of the hPXR. On the basis of its SR-EF, DIOP could be ranked amongst the most potent activators of hPXR as compared with the miscellaneous chemicals previously tested in the same cell line [12, 16, 17].

RP-HPLC fractionation of F3 allowed the isolation of PXR-like activity in fractions F3.28 to F3.34 (Table 4). These fractions were injected in GC-MS in full-scan mode but no peak was detected. Pre-fractionation calibration showed that the polar F3 pre-fraction may contain chemicals with log  $K_{OW}$  values of less than 3, such as steroids, BPA, and parabens (Tables 2 and 3). Therefore, it is likely that GC-MS was not suitable to identify such compounds due to their potential high polarity and/or low volatility properties. Several studies have reported that GC-MS offers a useful first approach for chemical identification due to the reproducibility of the mass spectra in electron impact ionization and the existence of a mass spectra databases [32]. However, the same authors highlighted that such a tool remains limited to the assessment of non polar and volatile chemicals. Hence, liquid chromatography coupled to high-resolution mass spectrometry (e.g., HR-LC-MS, LC-TOF, and LC-LIT-MS) or hybrid techniques (Q-TOF, LTQ-FT-Orbitrap®) will be required to identify hPXR activators in F3. The use of these techniques is increasingly reported for the identification of unknown polar compounds [33, 34].

#### Identification of ER ligands using an ER $\alpha$ affinity column

The strongest estrogenic activity was detected in F2.30, which exerted 36 % of the maximal response, but also to a lesser extent in F2.31 and F2.32 (Table 4). All of these fractions also possessed dioxin-like activity. In order to isolate ER ligands from AhR ligands, we used an hER $\alpha$  affinity column, which has been successfully used for the isolation of estrogenic activity in environmental matrices [20] or in infant formulas [35]. The three active fractions (F2.30–2.32) were pooled, deposited on the hER $\alpha$  affinity

**Table 4** In vitro RP-HPLC profiles of F2 and F3 SPE fractions of Réveillon sediment extract

RP-HPLC Fractions	SPE-F2				SPE-F3			
	ER	hPXR	AhR 24h	AhR 4h	ER	hPXR	AhR 24h	AhR 4h
f1		3						
f2								
f3								
f4								
f5								
f6					11			
f7					10			
f8					8			
f9		6						
f10								
f11								
f12								
f13								5
f14					6			
f15					16			
f16					47			
f17				5				
f18				42	13			
f19								
f20		5						34
f21								
f22		8						39
f23								47
f24	10			38		7		51
f25				27		5		58
f26				67		7		55
f27				58				49
f28			4	74		13		57
f29			6	74		21		42
f30	36		9	76		22		17
f31	9		7	78		23		
f32	9		4	80	5	31		
f33				72		16		
f34		6		47		13		
f35		14		6				29
f36		8						
f37								
f38				16				
f39								
f40	15							

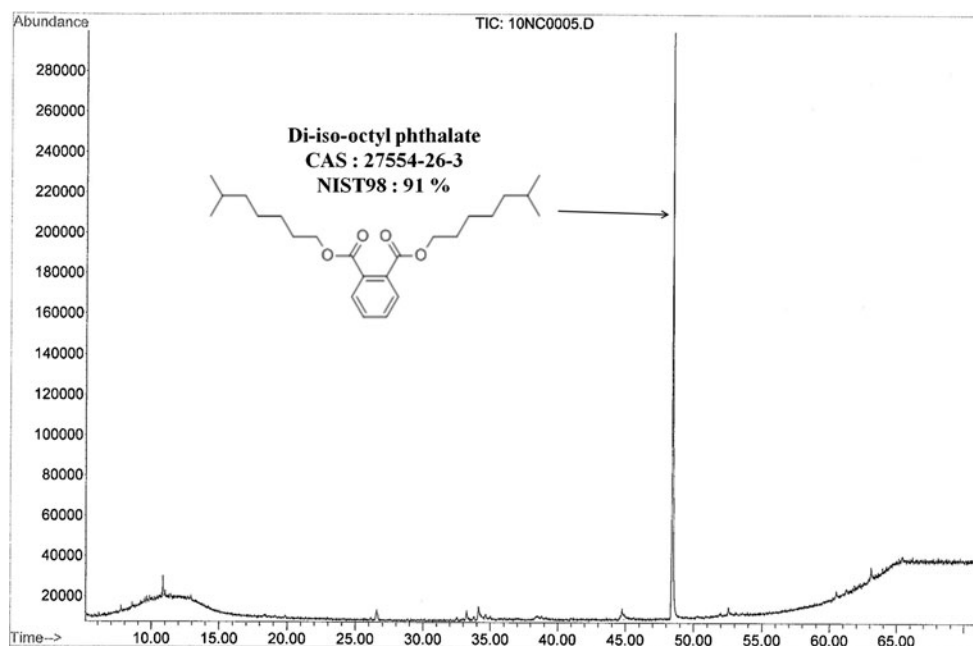
	No or weak effect ( $\leq 10\%$ <sup>a</sup> )
	Moderate effect (10-50% <sup>a</sup> )
	Strong effect (>50% <sup>a</sup> )

<sup>a</sup> Results are expressed as a percentage of luciferase or EROD activity relative to maximal induction by the reference compound

column. Fractions corresponding to FT, washing (L1–L3) and elution (E1–E4) steps were then collected and tested for biological activities. Analysis of bioassays showed that estrogenic activity was only detected in the elution

fraction E2 while dioxin-like activity was detected in FT and thus not retained by the column (data not shown). This elution fraction was then injected into GC-MS in full scan mode, allowing the identification of 2,4-di-*tert*-

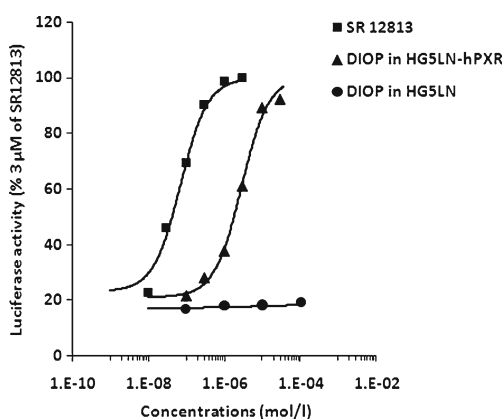
**Fig. 7** Total ion chromatogram of the F2.35 fraction



butylphenol (2,4-*t*-BP) and 2,6-di-*tert*-butyl- $\alpha$ -methoxy-*para*-cresol (DTB) as major compounds (Fig. 9). Alkylphenols have been widely described as endocrine disruptors through their capacity to bind to and activate the ER. Tollefsen et al. [36] have recently described the capacity of 2,4-*t*-BP to bind to the rainbow trout ER while Sun et al. [37] demonstrated that 4-*t*-butylphenol activates rat and human ER. To our knowledge, the estrogenic effect of DTB has not been described previously. Butyl-hydroxy-toluene (i.e., BHT or 2,6-di-*tert*-butyl-*para*-cresol) and *p*-cresol, which are structurally closed, were described as human ER ligands [38, 39]. The potency of 2,4-*t*-BP and DTB to induce luciferase

activity in MELN cells has been further confirmed using pure chemicals (Fig. 10).

Estrogenic activity was also detected in F3.6–8, F3.15–16, and F3.18 (Table 4). SPE-HPLC calibration showed the elution of standards *n*-propylparaben in F3.15, *n*-butylparaben in F3.18 and BPA and  $\beta$ E2 in F3.16 (Table 3). Kinani et al. reported the occurrence of *n*-propylparaben, *n*-butylparaben, BPA, and  $\beta$ E2 in this river sediment [13]. In the present study, targeted chemical analyses confirmed the occurrence of BPA and  $\beta$ -E2 in F3.16 (data not shown). As already observed in crude extract, BPA weakly contributed to estrogenic response in F3.16 whereas  $\beta$ -E2 explained 50 % of the estrogenic activity in this fraction (data not shown).

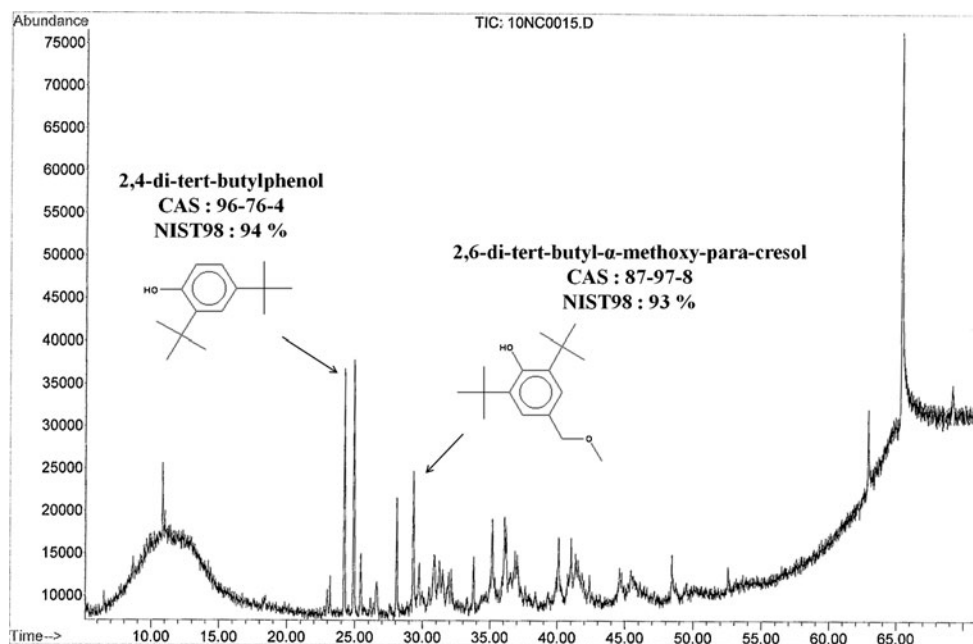


**Fig. 8** Dose response curves of di-iso-octylphthalate (DIOP) in HG5LN-hPXR and HG5LN cells. Results (mean $\pm$ SD of triplicates) are expressed as a percentage of luciferase induced by 3  $\mu$ M of SR12813

#### Other biological activities

Surprisingly, no anti-androgenic activity could be detected in any of the RP-HPLC fractions (data not shown). Nevertheless, the pool of the 40 fractions exerted significant anti-androgenic activity, which was similar to that of the initial F2 (Fig. 6b). This suggested that overall anti-androgenic activity was due to many different chemicals that were distributed over many different fractions, hence impeding their detection in individual fractions. This demonstrated that fractionation strategy remains a critical task when several biological activities are to be considered. To date, only few studies have dealt with EDA an approach using more than two biological endpoints [7, 8, 40]. These studies showed that a reduced number of fractions (3 to 20) often led to the co-occurrence of several biological activities in the

**Fig. 9** Total ion chromatogram of pooled F2.30–32 fractions after purification on an hER $\alpha$  column



same fraction, hence rendering difficult the specific identification of the active chemicals. In the present case study, further investigations, for instance using normal phase-based HPLC fractionation step, will be necessary in order to identify anti-androgenic chemicals.

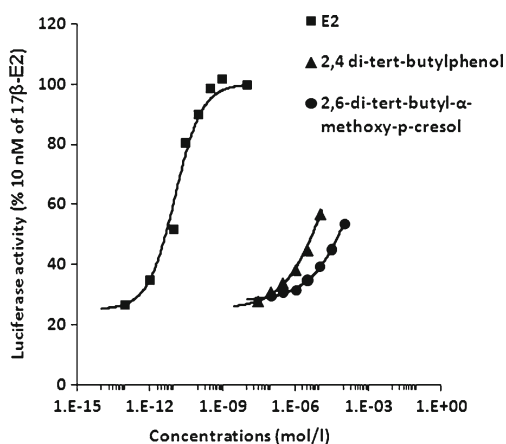
Regarding dioxin-like activity, it was distributed between many successive fractions yielding a cloudy signal rather than clear isolated peaks. Such a pattern could be explained by either the occurrence of numerous AhR activators that were eluted in different fractions or the occurrence of few AhR ligands with an overlapping elution profile in these fractions. This may suggest that the established RP-HPLC protocol was not adapted to

the isolation of AhR ligands. However, Luebcke-von Varel et al. [7] using calibrated NP-HPLC observed a similar display of active fractions.

## Conclusions

Our study demonstrated the usefulness of the combined use of multi-receptors and multi-step fractionation approaches to reveal a broad range of EDCs in complex environmental samples. Here, our approach allowed the successful isolation and identification of novel hPXR and ER ligands. In addition, the NR affinity column appears as a useful tool for the final purification of active estrogenic chemicals in case of multiple biological activities. One significant result was the identification of di-iso-octyl-phthalate as a strong inducer of hPXR.

Nevertheless, our results also revealed some limitations of the method as observed with the loss of anti-androgenic activity or the spreading of the dioxin-like activity in the fractions. The fractionation strategy should be considered as a dynamic process that should be modulated for the isolation of active chemicals. Moreover, the use of an NR affinity column on the primary fractions would also provide a powerful alternative to the isolation and identification of new EDCs such as AR or hPXR ligands [41]. Overall, several fractionation tools can be used alone or in combination for the isolation of a broad range of active chemicals in order to enhance knowledge on the occurrence of EDCs in situ.



**Fig. 10** Dose response curves of 2,4-di-tert-butylphenol and DTB in MELN cells. Results are expressed as a percentage of maximal luciferase induction by 10 nM of 17 $\beta$ -E2

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