Supporting information

Method development for effect-directed analysis of endocrine disrupting compounds in human amniotic fluid

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15 pages containing 7 figures and 2 tables

S1. – Chemicals

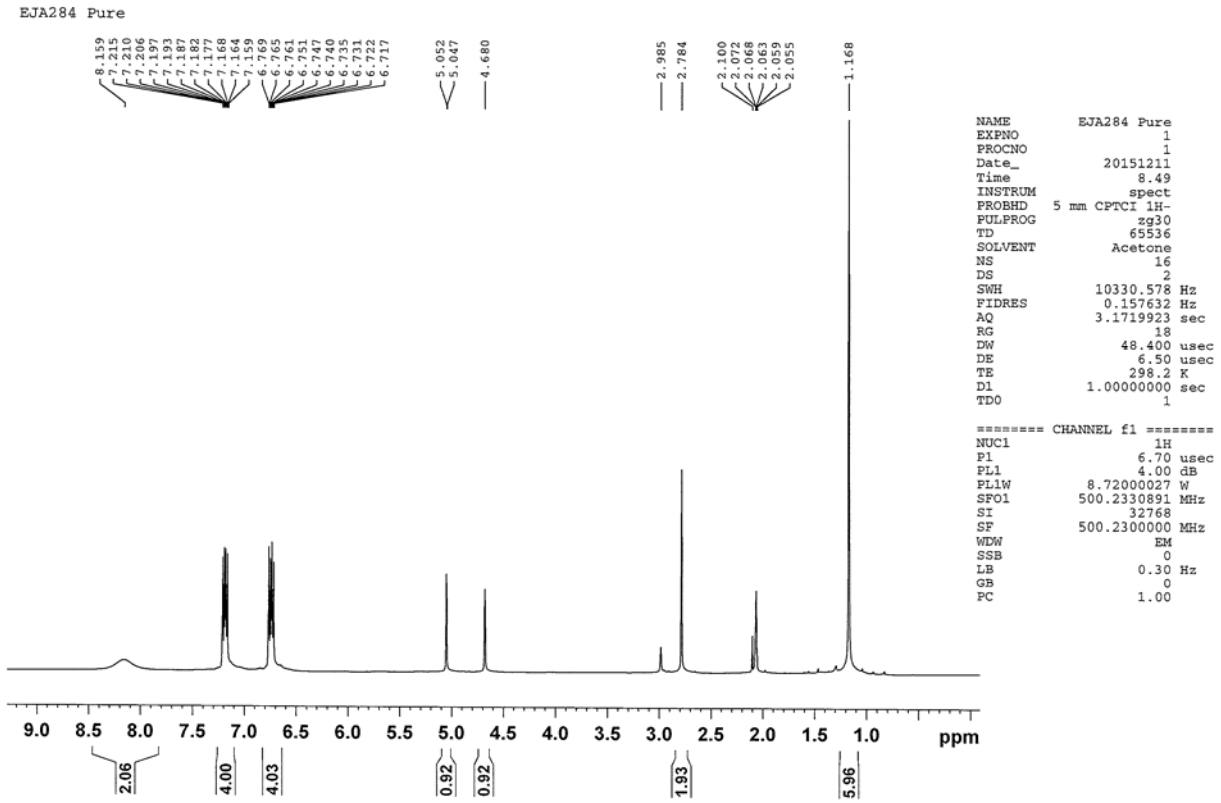
α-Minimal Essential Medium (MEM) with GlutaMax (32561-029), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (DMEM/F12) with GlutaMax (31331-028), DMEM/F12 medium without phenol red (11880-028), Leibovitz's L-15 medium (L-15, 11415-049), L-15 medium without phenol red (21083-027), Hank's Balanced Salt Solution (HBSS, 14175-053), penicillin/streptomycin (P/S, 15140-122), trypsin (25300-054) and foetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany); G418 (ant-gn-1, ant-gn-5, 10832-42-2) was obtained from InvivoGen (Toulouse, France); Steady-Glo® Luciferase Assay system (E2550) was purchased from Promega (Leiden, The Netherlands). Methanol (MeOH) and dichloromethane (DCM) were purchased from Thermo Fisher Scientific (Loughborough, UK); acetone was obtained from BDH Laboratory (Poole, Dorset, UK); dimethyl sulfoxide (DMSO, 99.9+%) was obtained from Alfa Aestar (MA, USA). All solvents used were of HPLC grade. Ammonium hydroxide solution (ACS reagent, 28.0-30.0% NH3 basis) was purchased from Sigma Aldrich (Poole, Dorset, UK); LC-MS grade water (LiChrosolv®) was obtained from Merck KGaA (Darmstadt, Germany); chloroform (ACS reagent, 99.8+%) was purchased from Acros Organics (Geel, Belgium). Two hydroxylated metabolites of brominated flame retardants, namely 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47) and 3-hydroxy-2,2',4,4',5,5'-tetrabromodiphenyl ether (3-OH-BDE-153) were generously provided by Dr. Lillemor Asplund (Stockholm University). All other reference compounds were purchased from Sigma Aldrich (Poole, Dorset, UK) at the highest commercially available purity. 4-methyl-2,4-bis(4-hydroxyl- phenyl) pent-1-ene (MBP), a potent metabolite of bisphenol A, was synthesized and its identity and purity was confirmed spectrometrically (NMR, IR and HR-MS) as described below.

S1.1 – Synthesis of 4,4'-(4-methylpent-1-ene-2,4-diyl)diphenol (MBP)

Materials and Methods: NMR spectra were recorded on a Bruker Avance 500 (125.78 MHz for ^{13}C) using the residual solvent as internal standard (^{1}H: δ 2.05 ppm, $^{13}C\{^{1}H\}$: δ 206.26 ppm for acetone-d_6. Chemical shifts (δ) are given in ppm and coupling constants (*J*) are given in Hertz (Hz). Melting points were recorded on a Büchi M-565 melting point apparatus. Electrospray Ionization (ESI) high resolution mass spectroscopy was carried out using a Bruker micrOTOF-Q instrument in negative ion mode. Infrared spectra were recorded neat using a Shimadzu FTIR-8400s spectrophotometer and wavelengths are reported in cm^{-1}. Flash chromatography was performed on Silicycle Silia-P Flash Silica Gel (particle size 40-63 μm, pore diameter 60 Å) using the indicated eluent. Thin layer chromatographic (TLC) analysis was performed with Merck F254 silica gel-60 and visualized by UV light.

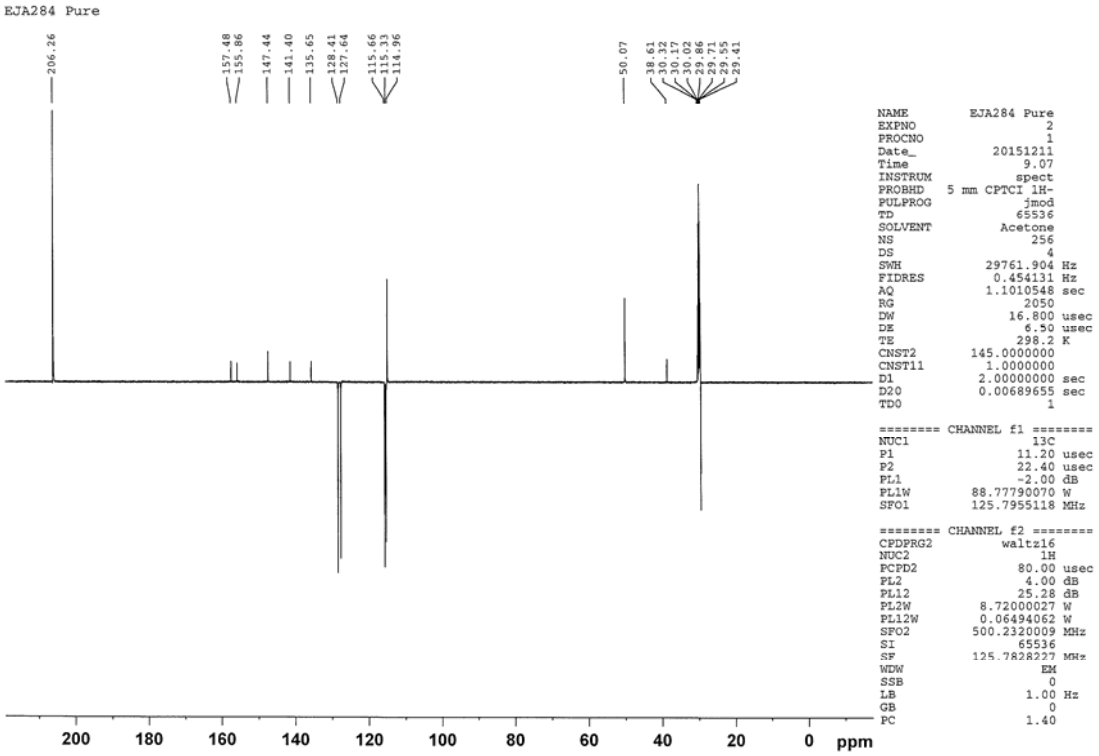
4-Methyl-2,4-bis-(p-hydroxyphenyl)pent-1-ene (MBP): Bisphenol A (2.00 g, 8.76 mmol) was dissolved in concentrated sulfuric acid (6.86 mL) and the orange solution was poured over ice water (116 mL) under vigorous stirring. The resulting solids were collected by filtration. The filtrate was washed with MTBE (3 x 20 mL) and the previously obtained solids were dissolved in the combined organic layers. Traces of sulfuric acid in the organic layer were neutralized with saturated aqueous $NaHCO_3$, after which the organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The residue was purified by flash silica gel chromatography (gradient, CH_2Cl_2:EtOAc 97.5:2.5 → 90:10) to give MBP (235 mg, 20 %) as a white solid.

Figure S1.1 - Proton NMR spectrum of synthesized MBP



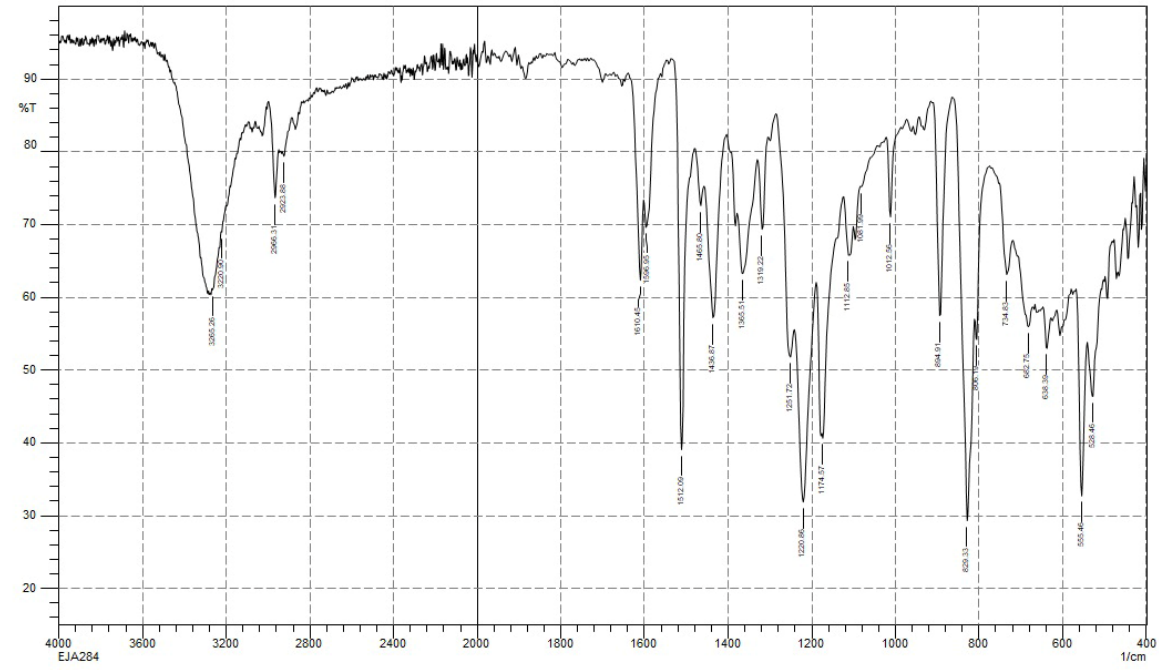
^{1}H NMR: (500 MHz, Acetone-d_6) δ 8.16 (br s, 2H), 7.19 (ddd, 4H, J = 11.0 Hz, 9.0 Hz, 2.5 Hz), 6.74 (ddd, 4H, J = 11.0 Hz, 9.0 Hz, 2.0 Hz), 5.05 (d, 1H, J = 2.5 Hz), 4.68 (s, 1H), 2.78 (s, 2H), 1.17 (s, 6H);

Figure S1.2 - ^{13}C NMR spectrum of synthesized MBP



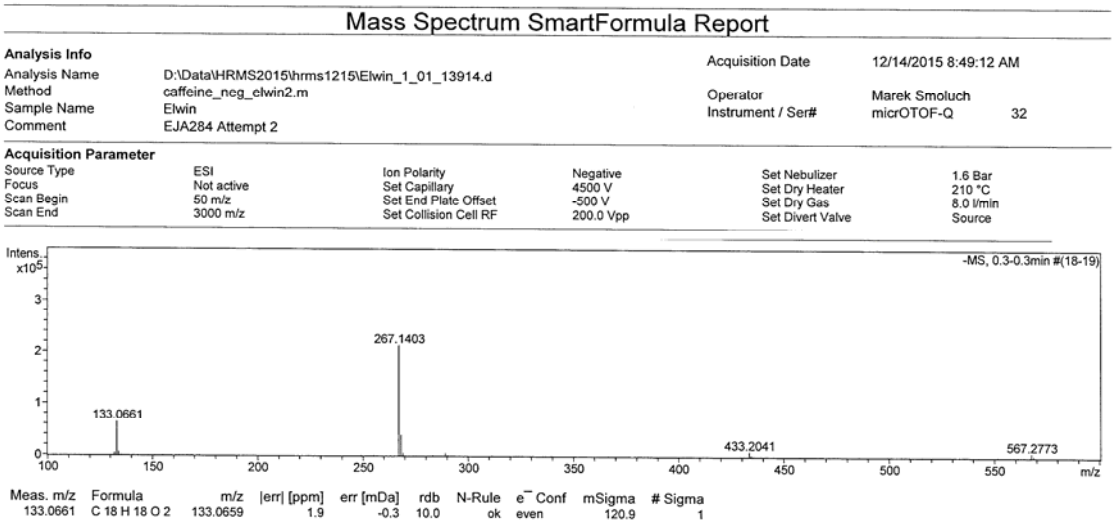
^{13}C NMR: (125 MHz, Acetone-d_6) δ 157.5, 155.9, 147.4, 141.4, 135.7, 128.4 (2C), 127.6 (2C), 115.7 (2C), 115.3 (2C), 115.0, 50.1, 38.6, 29.4 (2C);

Figure S1.3 - Infra Red (IR) spectrum of synthesized MBP



IR (neat) vmax (cm^{-1}): 3265 (s), 1610 (m), 1512 (s), 1437 (m), 1366 (m), 1221 (s), 1175 (s), 895 (m), 829 (s), 555 (s);

Figure S1.4. - HRMS (ESI) spectrum of synthesized MBP



HRMS (ESI): *m/z* calculated for $C_{18}H_{19}O_2$ $[M\text{-}H]^-$ 267.1391, found 267.1403, $C_{18}H_{18}O_2$ $[M\text{-}2H]^{2-}$ 133.0659, found 133.0661; melting point 127.3-129.5 °C.

Table S1. Concentration of the reference compounds in the spike mix used during method development of extraction and fractionation of amniotic fluid.

Compound	Concentration in the spike mix (µg/mL)
4-OP	0.788
PP	0.394
MnBP	0.708
BPA	0.570
BPS	0.679
MBP	0.336
TCS	0.423
OH-PCB-61	0.729
PFOS	0.263
6OH-BDE-47	0.378
3OH-BDE-157	0.741
daidzein	0.705
genistein	0.188
enterolactone	0.308
FICZ	0.345
E2	0.693
E1	0.292
E4	0.797

S2 - Extraction procedures

Solid phase extraction (SPE): MCX columns (Oasis, 6 cc, 150 mg/3 mL, 30 µm particle size, Waters Corp., Milford, MA) and HLB columns (Oasis, 6 cc, 150 mg, 30 µm particle size, Waters Corp., Milford, MA) were conditioned with 3 mL of methanol and 3 mL of deionised water. Matrix (5ml of LC-MS grade water or AF) was spiked with the reference mix (100 µL), pH was adjusted to 2 with 98% formic acid (for MCX extraction only), and then samples were loaded on the cartridges at a rate of 1 mL/min. The MCX and HLB cartridges were rinsed with 3 mL of 2% formic acid (in deionized water) and with 5% methanol respectively, and then air dried for 1 min. The analytes were eluted from the MCX cartridges with 2 x 2 mL of 100% methanol and 2 x 2 ml of 5% of ammonium hydroxide, and from the HLB cartridges with 4 x 2ml of 100% methanol. Deionised water (0.2 mL) was added to the extracts and the extracts put in a water bath (max. temperature 40°C) where the solvent was evaporated under a gentle stream of nitrogen to a final volume of 0.2 mL. The samples were then reconstituted with 0.3 mL of 10% methanol in deionised water, transferred to glass vials for analysis and analysed on LC-ESI-MS/MS for chemical recoveries.

Dispersive liquid/liquid extraction (DLLE): 5 ml of LC-MS grade water or amniotic fluid was spiked with the reference mix (100 µL) and pH adjusted to ~4.5 with sodium acetate/acetic acid buffer (0.1 M). DLLE was performed by addition of DCM (0.5 mL) or chloroform (0.5 mL) only, or by addition of a mixture of acetone (1ml) (dispersive solvent) and DCM (0.5 mL) or chloroform (0.5 mL) (extraction solvent). Samples were then vortexed 2 x for 15 seconds and centrifuged at 4081 x g for 15 min at 25°C. The supernatant was removed, 50 µL of deionised water was added, the organic layer evaporated under gentle stream of nitrogen and the residue reconstituted with 10% MeOH to a final volume of 0.5 mL. Samples were transferred to glass vials and analysed on LC-ESI-MS/MS for chemical recoveries.

Table S2. Parameters used during LC-ESI-MS/MS Multiple Reaction Monitoring (MRM) detection of the analytes including collision cell parameters and monitored precursor/product ions. Limit of detection (LOD) for each analyte was calculated as described in the method section.

Compound	Q1	Q3a	Q3b	DT	EP	DP (Q/C)	CE (Q/C)	CXP (Q/C)	LOD (M)
4-OP	205.18	106.0	189.0	25	-10	-55	-28	-15	4.4×10^{-10}
PP	179.13	91.9	136.0	25	-10	-55	-32	-13	6.0×10^{-11}
MnBP	221.05	121.1	70.9	25	-12	-80	-20	-13	$2.0. \times 10^{-10}$
BPA	227.20	133.1	212.0	25	-11	-90/-90	-40/-26	-13/-13	3.7×10^{-09}
BPS	249.10	107.9	156.0	25	-10	-70	-38	-17	3.7×10^{-11}
MBP	267.20	133.1	117.2	25	-11	-100/-150	-25/-50	-13/-15	9.4×10^{-12}
TCS	287.09	34.9	241.1	25	-10	-40	-48	-7	5.4×10^{-11}
OH-PCB-61	306.95	270.8	34.9	25	-10	-55/-55	-30/-82	-19/-17	9.9×10^{-11}
PFOS	498.95	79.9	98.9	25	-10	-170	-84	-17	9.9×10^{-12}
6OH-BDE-47	500.75	78.8	80.8	25	-10	-75	-40	-11	2.7×10^{-11}
3OH-BDE-157	658.55	80.8	576.6	25	-10	-105/-105	-100/-38	-13/-41	5.8×10^{-12}
daidzein	253.14	132.0	223.0	25	-10	-90/-90	-54/-36	-7/-13	1.7×10^{-11}
genistein	269.10	133.1	63.1	25	-10	-135/-135	-46/-50	-11/-11	2.9×10^{-11}
enterolactone	297.16	253.0	107.0	25	-10	-110/-150	-28/-50	-17/-15	2.7×10^{-11}
FICZ	283.30	254.2	255.3	25	-10	-90	-40	-13	2.1×10^{-11}
E2	271.15	183.0	145.0	25	-10	-80/-50	-56/-56	-11/-17	4.1×10^{-11}
E1	269.12	145.0	142.9	25	-10	-105	-52	-23	1.6×10^{-11}
E4	303.12	241.0	273.0	25	-14	-90	-28	-15	3.8×10^{-11}

Q1, precursor ion (m/z); Q3a, quantification product ion (m/z); Q3b, confirmation product ion (m/z); DT, dwell time (ms); DP, declustering potential (V); EP, entrance potential (V); CE, collision energy (V); CXP, collision cell exit potential (V)

S3 – Cell culture

The estrogen receptor-mediated luciferase reporter gene (ER-Luc) human breast carcinoma (VM7Luc4E2) cells[1], the androgen/glucocorticoid receptor-mediated luciferase reporter gene (AR-Luc) human breast carcinoma (MDA-kb2) cells[2] and the aryl hydrocarbon receptor enhanced green fluorescent protein reporter gene (DR-GFP) mouse hepatoma (H1G1.1c3) cells[3] were routinely maintained in 75 cm^2 canted neck tissue culture flasks (Greiner Bio-One Ltd.). ER-Luc and DR-GFP cells were maintained in DMEM/F12 and α-MEM medium respectively, supplemented with 10% FCS and 1% P/S (culture media) and incubated in a humidified incubator at 37 °C and 5% CO_2. DR-GFP cells were additionally supplemented with 1% G418. AR-Luc cells were maintained in Leibowitz-15 (L-15) medium supplemented with 10% FCS (culture media) and incubated in a humidified incubator at 37 °C without additional CO_2. Cells were subcultured when 70-80% confluent.

ER, AR and DR reporter gene assays

ER-Luc: Prior to experiments, ER-Luc cells were maintained in DMEM/F12 medium without phenol red (assay meadia) supplemented with 10% charcoal dextran stripped FCS (cdFCS) and 1% P/S for two media-changes (4 days). Cells were seeded at a density of $2x10^4$ cells per well in 100 µL of assay media in clear-bottomed 96-well tissue culture dishes (Greiner Bio-One Ltd.) and allowed to attach. After 24 h media was removed and 100 µL of assay media containing test chemicals or DMSO control (0.5%) was added to the wells. After 24h of exposure, media was removed, cells washed with PBS (200 µL), PBS removed and lysis buffer added (20 µL). After cell lysis (15 min with shaking) the luciferase activity was determined in a plate reader (LUMIstar Optima, BMG Labtech GmbH) with 100 µL of luciferase reagent media injected directly to each well during measurement.

AR-Luc: Prior to experiments, AR-Luc cells were maintained in L-15 medium supplemented with 10% cdFCS (pre-assay media) for two media-changes (4 days). Cells were then seeded at a density of $2x10^4$ cells per well in 100 µL of phenol red-free L-15 medium supplemented with 10% cdFCS (assay media) into white, clear-bottomed 96-well tissue culture dishes and allowed to attach. After 24 h, 100 µL assay medium was added to the wells with twice the intended final concentration of test chemicals or DMSO control (0.5%), to obtain the desired test concentrations. After 24 h of exposure, 100 µL of media was removed and SteadyGlo assay reagent (Promega UK Ltd.) (100 µL) was added to the wells and the plate left shaking for 10 min. The luciferase activity was determined as described above.

DR-GFP: DR-Luc cells were maintained in culture media and when confluent seeded in black, clear-bottomed 96-well tissue culture dishes (DR-Luc) (Gre- iner Bio-One Ltd.) in MEM Alpha media supplemented with 10% FCS, 1% P/S, but without addition of G418 (assay media). Cells were seeded at a density of $3x10^4$ cells per well in 100 µL of assay media and left to attach. After 24 h, media was aspirated and cells exposed to test chemical or DMSO control (1%) in 100 µL of assay media. After 24 h of exposure, fluorescence was measured in the intact cells (without removal of media) using a fluorescent plate reader (POLARstar Galaxy, BMG Labtech GmbH) with an excitation wavelength and an emission wavelength set at 485 nm and 510 nm, respectively. In order to normalize results the fluorescence gain was adjusted per plate to the highest activity of the positive control. In each assay the outer wells of the plate were not included in the measurements and were filled with 100 µL assay media only.

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, after 23 h exposure, media was removed from each well, 100 µl of 5 mg/ml freshly made MTT solution was added and plates were incubated at 37 °C. At t= 24 h, MTT solution was removed, lysis buffer (150 uL of isopropanol) was added to the

wells and the plate left shaking for 15 min. The absorbance at (λex = 590 nm) was measured with a POLARstar fluorescent plate reader.

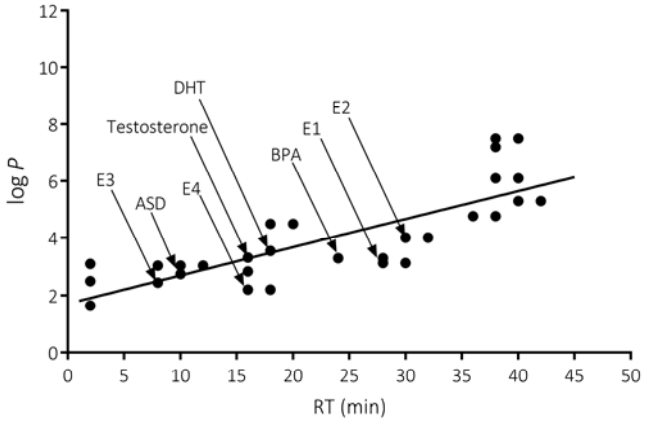


Figure S2. The log*P* of reference compounds retrieved from PubChem database (www.pubchem.ncbi.nlm.nih.gov) plotted against retention times (RT) during RP-HPLC fractionation of spiked AF extract, including estetrol (E4), 17β-estradiol (E2) estrone (E1) and bisphenol A (BPA). Log*P* of estriol (E3), androstenedione (ASD), dihydrotestosterone (DHT) and testosterone was plotted against their predicted RTs interpolated from linear regression analysis (GraphPad Software Inc., San Diego, CA).

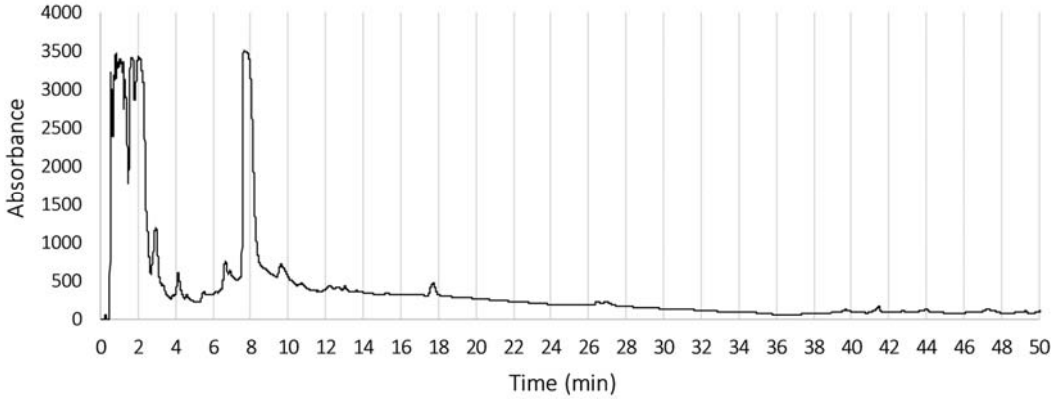


Figure S3. An example of a chromatogram obtained during reversed-phase fractionation of AF extract. Sample was injected onto reverse phase Phenomenex Kinetex C18 (100mm x 2.1 mm, 5 µm pore size) column using an Agilent 1260 system equipped with diode array detector (DAD). Absorbance was monitored with a 253 nm wavelenght and plotted against retention time. Eluted fractions were concentrated, resuspended in DMSO and used for bioassay analysis.

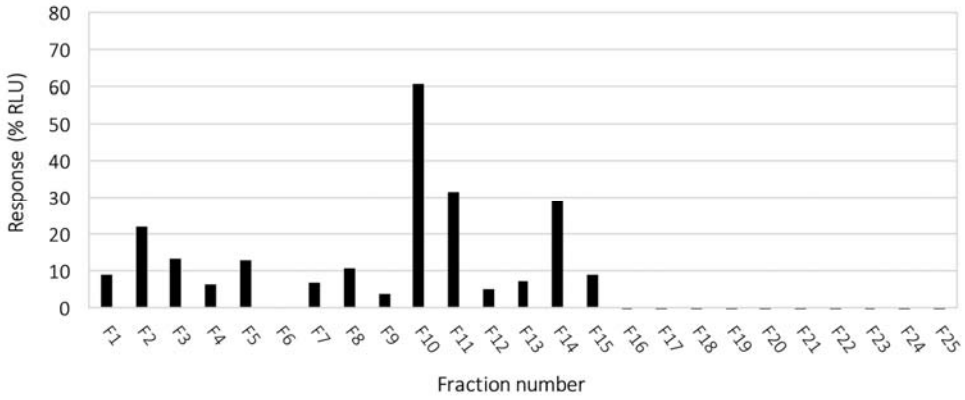


Figure S4. Agonistic activity of AF fractions (50 x diluted) in the ER-Luc assay. ER activity was measured as described in the method section (n=1).

References

(1) Rogers, J. M.; Denison, M. S. Recombinant Cell Bioassays for Endocrine Disruptors: Development of a Stably Transfected Human Ovarian Cell Line for the Detection of Estrogenic and Anti-Estrogenic Chemicals. *In Vitr. Mol. Toxicol.* **2000**, *13* (1), 67–82.

(2) Wilson, V. S.; Bobseine, K.; Lambright, C. R.; Gray, L. E. A Novel Cell Line, MDA-Kb2, That Stably Expresses an Androgen- and Glucocorticoid-Responsive Reporter for the Detection of Hormone Receptor Agonists and Antagonists. *Toxicol. Sci.* **2002**, *66* (1), 69–81. https://doi.org/10.1093/toxsci/66.1.69.

(3) Nagy, S. R.; Sanborn, J. R.; Hammock, B. D.; Denison, M. S. Development of a Green Fluorescent Protein-Based Cell Bioassay for the Rapid and Inexpensive Detection and Characterization of Ah Receptor Agonists. *Toxicol. Sci.* **2002**, *65* (2), 200–210. https://doi.org/10.1093/toxsci/65.2.200.