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Detection and elimination of human exposure to environmental hormone disrupting substances.

LC/MS method1 protocol

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Experimental procedure LC/MS method1.

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A Chemicals and reagents

17β–estradiol, estrone, estriol, 17α-ethynylestradiol, and diethylstilbestrol were a kind gift of Professor Van Den Bossche of the Laboratory for Pharmaceutical Chemistry and Drug Analysis (Ghent University, Belgium). The internal standard, a deuterated form of 17β–estradiol (1,3,5[10]-estratriene-2,4-d2-3,17β–diol), was purchased from Sigma-Aldrich Chemicals (Bornem, Belgium). Ammonium acetate, formic acid, and acetic acid were purchased from Merck-Eurolab (Leuven, Belgium). Ammonium formate was obtained from Sigma-Aldrich Chemicals (Bornem, Belgium). Water, methanol, and acetonitrile were all of HPLC gradient grade (Merck-Eurolab, Leuven, Belgium). 6-mL Isolute® cartridges packed with 2 g of C18 material and a surface area of 521 m^2/g were purchased from Sopachem (Brussels, Belgium).

B Extraction procedure

An individual standard solution of 1 g/L of each estrogen was prepared in methanol and stored in the dark at –20°C until use. Under these conditions the solution proved stable for more than 6 months. Working solutions, containing a mixture of the estrogens in concentrations ranging from 40ng to 4µg/mL (50µL injected on column (oc)), were prepared by dilution in a mixture of water-acetonitrile (50:50, v/v).

River water samples (50mL) were collected from the river Schelde (Ghent, Belgium) and spiked with 20ng of the internal standard. The following simple solid phase extraction procedure was developed. The C18 SPE columns were activated using 4mL of methanol followed by 10mL of HPLC water. At a rate of approximately 10mL/min the sample was passed through the column after which the column was dried under vauum for 20 minutes. 3mL of aceton was used to elute our target compounds from the extraction column. The extract was dried under a gentle stream of nitrogen on a TurboVap® LV Evaporator (Zymark, MA, U.S.A.) and redissolved in a 50µL mixture of water-acetonitrile (50:50, v/v) and a 25-µL aliquot was injected. For recovery experiments, a portion of the free estrogens was spiked into the samples.

C Chromatographic conditions

Chromatography was carried out using a LaChrom® separation module (Merck, Darmstadt, Germany) including a L-7100 Low-Pressure Gradient Pump, L-7200 Autosampler (injection loop 100µL), L-7360 Column Oven, and D-7000 Interface. The system uses the LC/3DQ-MS System Manager Software running under Windows NT ™ version 4.0 on a Compaq Deskpro EN.

All estrogens could be separated on a Purospher® Star RP-18 column (55 x 2 mm I.D., particle size 3µm) using a gradient system at a flow rate of 250µL/min. Mobile phases consisted of water (A) and acetonitrile (B) with no buffer added. The gradient profile started with a short isocratic period of 20% acetonitrile. At 0.2 min the percentage of organic modifier was increased op to 54% where it was held for 12 min. The gradient increased linearly to 95% acetonitrile in 2 min and was brought back to starting conditions in another 2 min. Under these conditions the last peak (DES isomer) eluted within 12 min. Between two runs a 5 minute equilibration time was incorporated.

D Mass spectrometry

All MS experiments were carried out on the M-8000 ion-trap based mass spectrometer from Merck (Darmstadt, Germany) equipped with a pneumatically assisted electrospray or ionspray (IS) interface, on-axis with the sampling orifice of the mass spectrometer, and operated in negative ion mode. All experiments were performed using the 'Automatic Sensitivity Control' (ASC) facility to automatically adjust the accumulation time as the ion abundance changed. Instrument control, data acquisition, and data control were performed using the same software as for the HPLC. The direct transfer from the obtained data to Excell is performed by 'Dynamic Data Exchange' (DDE), a special function incorporated in the LC/3DQ-MS System Manager Software.

Optimization of the settings of the ion source and the ion-trap based mass analyzer as well as evaluation of the influence of the eluent composition were performed by flow injection analysis (FIA). By installing a syringe pump (Harvard Apparatus, Hollington, MA) and a LC gradient pump in parallel, connected by means of a stainless steel T-piece to the mass spectrometer, and running a gradient with the test solvent, the effect of an increasing amount of the evaluated solvent on the ionization efficiency of the interface for our test compounds could be clearly visualized. The syringe pump delivered a direct and continuous flow (25µL/min) of estradiol (20µg/mL) to the MS, while the flow rate of the gradient pump was fixed according to optimal settings advised by the manufacturer, namely 225µL/min. The gradient program started with 100% A (starting solvent), changed linearly to 100% B (test solvent) in 20 minutes and stayed there for 10 minutes before returning to the starting conditions (100% A). This condition was held for another 10 minutes to check for the complete removal of the test solvent from the system. Experiments were performed three times to make sure the observed phenomena were correct and not influenced by previous tests. Table B.4.3-1 lists the solvent compositions used in each individual experiment.

Table 1: Overview of the different solvent compositions applied in the gradient program (all mixtures represent v/v ratios)

Parameter	A	B
Organic modifier		
Methanol	Water	Methanol
Acetonitrile	Water	Acetonitrile
Volatile base		
ammoniumhydroxide	Water/acetonitrile (50:50)	0.1vol% ammoniumhydroxide in A
Isopropylamine	Water/acetonitrile (50:50)	0.1vol% Isopropylamine in A
Triethylamine	Water/acetonitrile (50:50)	0.1vol% Triethylamine in A
Buffer		
Ammonium formate (AmF)	Water/acetonitrile (50:50)	50mM AmF in A
Ammonium acetate (AmA)	Water/acetonitrile (50:50)	50mM AmA in A

The IS interface parameter settings were as follows: auxiliary gas heater: 600°C, capillary voltage: 2 kV, drift: 70 V, focus: 30 V, aperture 1 temperature: 150°C, aperture 2 temperature: 120°C, and nitrogen gas flow at 300 kPa. Standard MS parameters were operated at: accumulating masses: 200 to 350 amu, accumulation voltage: 0.08 V, low mass cutoff: 100 amu, and scan range 100 to 350 amu. High purity helium was used as a buffer gas to trap ions in the mass analyzer at 260 kPa (supply pressure to the ion trap's capillary). The detector voltage (photomultiplier, scintillator, conversion dynode, and direct current amplifier) was set at 450 V.