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ANALYSIS OF ENDOCRINE DISRUPTING COMPOUNDS IN WASTEWATER AND DRINKING WATER TREATMENT PLANTS AT THE NANOGRAM PER LITRE LEVEL

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ABSTRACT

The determination of steroid hormones, alkylphenolic compounds and bisphenol A at the ng l^{-1} level in environmental water samples (surface water and WasteWater Treatment Plant samples (WWTP)) is performed by a specific analytical procedure. Pre-concentration by solid-phase extraction conditions was optimized using C18 cartridges for steroid hormones and polymeric Oasis HLB cartridges for phenolic compounds. Identification and quantification were performed using a LC-MS/MS system with electrospray ionization in the negative mode for both compound families. For steroid hormones, the need to have limits of detection lower than 0.5 ng l^{-1} in WWTP samples led to the improvement of a purification step on silica cartridges. In the case of the phenolic compounds, no purification was required because of their lower estrogenicity. The limits of detection in WWTP effluents ranged between 0.02 ng $l^{-1}$and 0.21 ng l^{-1} for steroid hormones and 0.4 and 10.2 ng l^{-1} for phenolic compounds. The method was then applied to determine concentrations of the target compounds at each step of a WWTP. The process efficiencies were evaluated. Finally, concentrations were measured in influents and effluents of a Drinking Water Treatment Plant showing the complete removal of estrogenicity.

Keywords: Endocrine disrupters, LC-MS/MS analysis, wastewater treatment plant, drinking water

INTRODUCTION

In the past few years, the scientific community has shown a growing interest in environmental contaminants that can interfere with the normal endocrine function of wildlife and possibly humans [1-8]. Endocrine disrupting compounds (EDCs) can exert their effects by different mechanisms: by mimicking or antagonizing the effects of hormones, by altering the pattern of synthesis and metabolism of hormones and by modifying hormone receptor levels. Due to the large variety of suspected EDCs, it is probable that humans and animals are exposed not to a single agent, but to a mixture of multiple endocrine disrupting agents [9]. EDCs are suspected of entering rivers, streams and surface waters through the effluents of wastewater treatment plants (WWTP), showing that treatment efficiency is only partial. Several studies have shown the presence of EDCs in influents and effluents of WWTP in different industrialized countries [10-13]. Steroid hormones, alkylphenolic compounds and bisphenol A are representative EDCs found in WWTP effluents and information about their behavior during the treatment is needed.

Natural and synthetic hormones, such as estrone (E1), 17 β-estradiol (E2), 16 α-hydroxyestrone (OHE1) and 17 α-ethynylestradiol (EE2), are extremely potent estrogen receptor modulators [14-19]. Purdom *et al*. [17] have shown that a concentration of 1 ng l^{-1} of EE2 is sufficient to cause an alteration in the reproductive system of fish exposed to WWTP effluent. Alkylphenolic compounds, such as 4-*n*-nonylphenol (NP) or 4-*tert*-octylphenol (OP), are derivative of non-ionic surfactants used commercially for many years as household cleaning products. Their concentration levels in the environment are greater than those of hormones and their estrogenicity in fish has been shown [20-22]. Bisphenol (BPA) is an estrogenic, hormonally active monomer widely employed in the manufacture of consumer products such as epoxy resins, flame retardants and polycarbonates. This extensive use of BPA creates waste

during handling and other releases and has led to some concern regarding the potential deleterious effects of this compound, and similar compounds, on human health [23-24].

Monitoring these compounds in water is of great importance and studies have been conducted in order to quantify them at very low levels. Most of these studies have relied on a solid-phase extraction (SPE) followed by a derivatization step prior to detection by gas chromatography-mass spectrometry (GC-MS) [25-29]. Kuch and Ballschmiter [30] have determined phenolic compounds and estrogens in surface and drinking water at the pg l^{-1} level by high-resolution gas chromatography with negative chemical ionization mass spectrometric detection (HRGC-(NCI)-MS). Liquid chromatography-tandem mass spectrometry (LC-MS) can be used for a wide range of molecules and matrixes without derivatization. Steroid hormones and alkylphenols have already been determined using LC-MS with electrospray ionization in negative mode [31-34]. Lagana *et al*. [9] have improved a method using LC-MS/MS for assessing the occurrence of trace amounts of 12 representative estrogenic compounds in sewage and surface waters. Limits of detection (LOD) ranges in WWTP effluent were 0.8-1.1 ng l^{-1} and 1.0-55.0 ng l^{-1} for steroid hormones and alkylphenolic compounds respectively. However, despite the recent progress in analytical chemistry, understanding the behavior of endocrine disrupting compounds in a WWTP remains important and the first approach is the determination of concentrations at each step of the treatment.

Thus, in this present work, a method is developed using LC-(ESI)-MS/MS in negative mode to determine some EDCs, four steroid hormones and five phenolic compounds, at the ng l^{-1} level in water samples with high levels of organic matter. First, LC-MS/MS conditions were studied and optimized. Then, two SPE procedures were developed to analyze estrogen steroids and alkylphenols with acceptable recoveries in WWTP samples. The treatment efficiency of a WWTP was investigated by measuring concentrations of the target compounds after each process. Finally, the absence of estrogenic compounds in drinking water was verified.

MATERIALS AND METHODS

Chemicals

All solvents used in this study were of analytical grade or residual pesticide grade. Acetonitrile and water were purchased from Merck (Fontenay sous Bois, France). Methanol, cyclohexane and ethyl acetate came from Carlo Erba (Val de Reuil, France). Water used in SPE was purified using a Milli-Q system.

Pure standards of steroid hormones (min. 98 %) were all purchased as powders from Sigma-Aldrich (Saint Quentin Fallaviers, France). Standards of 4-*n*-nonylphenol (99 %), 4-*tert*-octylphenol (99 %) and bisphenol A (98.5 %) were purchased as powders but the nonylphenoxy carboxylate (98 %) and the octylphenoxy carboxylate (98.5 %) were purchased in solution (acetone) at 10 mg.l^{-1} in 1 ml. All standards of alkylphenols and bisphenol A came from CIL Cluzeau (France).

Stock standard solutions for each powder were prepared at 1 mg l^{-1} in methanol and stored for six months at -20°C. Two working solutions containing all steroids and all phenolic compounds were freshly prepared for each experiment by appropriate dilution of the stock solution in a mixture of methanol-water (1:2, v/v).

Extraction

Samples from a domestic WWTP were preserved by storing them at 4°C in the dark immediately after sampling. Prior to extraction, samples with high levels of suspended matter were centrifuged at 4000 rpm for 10 min. To avoid clogging of the SPE cartridges, all environmental samples were filtered through glass microfiber filters (GF/F-0.7 μm pore size) obtained from Whatman (Mantes La Jolie, France). C18 Bond Elut cartridges (1 g) and Oasis HLB cartridges (200 mg) were purchased from Varian (Les Ulis, France) and Waters (Saint Quentin Yvelines, France), respectively. Extractions were carried out using an automated sample processor ASPEC XL (Automated Sample Preparation with Extraction Columns) connected to a syringe pump and a 15-channel valve selector from Gilson International (Roissy Charles de Gaulle, France). Some preliminary trials, which are not presented in this study, were carried out in order to determine the optimal conditions of extraction in terms of solvent flow, drying time and elution volume. Silica gel cartridges Bond Elut (1 g), used for steroid hormones in the purification phase in the case of complex matrixes, were obtained from Varian [35].

LC-MS/MS Analysis

Identification and quantification of the analytes were carried out using a Surveyor autosampler and LC pump coupled with a TSQ Quantum Discovery tandem mass spectrometer (Thermo Electron Corporation, Courtaboeuf, France). The column used for LC separation was a Purospher Star RP 18 (125 mm x 2 mm I.D., 5 μm, Merck). The column temperature was kept at 30 °C and the injection volume was 20 μl. A mobile phase gradient elution program with solvent A (methanol) and solvent B (water) was applied at a flow rate of 0.2 ml min^{-1}. The gradient program started with 40 % methanol and then increased to 80 % methanol in 1 min. After an isocratic period of 80 % methanol between 1 min and 3 min, the mobile phase A was linearly increased up to 100 % methanol from 3 min to 8 min. This condition was held until 16 min then the elution program was returned to initial conditions for 10 min.

The mass spectrometer was operated in negative mode electrospray ionization (ESI (-)). In order to optimize MS-MS conditions, full scan and product ion spectra for each compound were first acquired by infusion of 1 mg l^{-1} standard

solutions of each analyte. Collision energy and collision pressure were then finely optimized in tandem with the chromatographic conditions [33].

RESULTS

LC-MS/MS Conditions

First, the MS/MS conditions were studied. Fragmentation studies and optimization of spectrometric conditions were carried out by injection of 1 mg l^{-1} standard solutions of each analyte. Two major fragment ions were selected as shown in Table 1.

The quantification ion is the major fragment of the parent ion. To be sure that the analyzed substance has not been mistaken for another one, the presence of a second product ion was observed. Product ions for confirmation were not found in the case of nonylphenol and octylphenol.

Next, optimal LC conditions for the steroid estrogen analysis were examined. For the chromatographic separation, water-methanol and water-acetonitrile mobile phases, with buffers such as formic acid or ammonium acetate, were tested in order to evaluate the resolution, duration and sensitivity of the analysis. Based upon these criteria, the best overall results, which are not shown in this study, were achieved with the water-methanol mobile phase without buffer.

Solid Phase Extraction

Steroid hormones

For SPE experiments, 200 ml of Evian spring water, spiked at 250 ng l^{-1} with a mixture of the steroid estrogens of interest, was analyzed using C18 Bond Elut cartridges as specified in the experimental section. C18 cartridges were conditioned with 10 ml of acetonitrile and 10 ml of deionized water. The cartridges were loaded with 200 ml of sample in neutral conditions of pH, washed with 10 ml of deionized water and dried under nitrogen for 20 minutes. Elution was performed with 2 x 3 ml of acetonitrile and the extracts obtained were evaporated to dryness under nitrogen using a solvent evaporator. For WWTP samples, which contain high levels of organic matter and impurities, an additional purification was carried out on silica gel cartridges. The dried SPE extracts were reconstituted to 1 ml with a mixture of cyclohexane/ethyl acetate (9:1, v/v). Silica gel cartridges were conditioned with 5 ml of a mixture of cyclohexane/ethyl acetate (5:5, v/v) and 5 ml of a mixture of cyclohexane/ethyl acetate (9:1, v/v). The cartridges were slowly loaded with the reconstituted SPE extract and two elutions were carried out. In fact, the cartridges were first eluted with a mixture of cyclohexane/ethyl acetate (9:1, v/v) with the aim of removing impurities. Secondly, the cartridges were eluted with a mixture of cyclohexane/ethyl acetate (5:5, v/v) to recover the steroid hormones. The second fraction was then evaporated to dryness at 45°C under nitrogen, reconstituted to 1 ml with a mixture of methanol-water (1:2, v/v) and analyzed by LC-ESI-MS/MS.

Alkylphenols and bisphenol A

Two hundred ml of Evian spring water, spiked at 100 ng l^{-1} with a mixture of the phenolic compounds of interest, was analyzed using Oasis HLB cartridges as specified in the experimental section. Oasis HLB cartridges (200 mg) were conditioned with 10 ml of acetonitrile and 10 ml of deionized water. The cartridges were loaded with 200 ml of sample, previously adjusted to pH 5, washed with 10 ml of a mixture of methanol/deionized water (1:9) and dried under nitrogen for 20 min. Elution was performed with 2 x 3 ml of acetonitrile and the extracts obtained were evaporated to dryness under nitrogen using a solvent evaporator. For WWTP samples the same protocol was used, without additional purification.

Table 1. MS/MS conditions.

Compound	Abbreviation	$[M-H]^-$ Parent ion (m/z)	Product ion for quantification (m/z)	Collision energy (eV)	Product ion for confirmation (m/z)	Collision energy (eV)
Estrone	E1	269.1	145.0	38	143.0	51
17 β-estradiol	E2	271.1	183.0	47	253.0	34
17 α-ethynylestradiol	EE2	295.1	145.0	41	267.1	26
16 α-hydroxyestrone	OHE1	285.1	145.0	40	143.0	55
Nonylphenol	NP	219.1	106.0	21	-	-
Nonylphenol carboxylate	NPEC	277.1	219.1	20	133.0	42
Octylphenol	OP	205.1	133.0	22	-	-
Octylphenol carboxylate	OPEC	263.1	205.1	20	106.0	30
Bisphenol A	BPA	227.1	212.0	17	133.0	27

Evaluation of the Performances

Extraction

A mean recovery R was calculated from 2 recoveries (R1 and R2) evaluated at a low level (10 ng l^{-1}) and a high level (250 ng l^{-1} for steroid hormones and 100 ng l^{-1} for phenolic compounds) of the calibration range, respectively. R1 and R2 were determined by repeating the analytical procedure 3 times at each level and the Relative Standard Deviation (RSD) was determined to evaluate the constancy of the optimized protocol (RSD cannot exceed 20 %). Table 2 shows the recoveries obtained in spring water and WWTP effluents for steroid hormones and phenolic compounds.

For all steroid hormones, recoveries were acceptable for both matrices. Relative standard deviation values did not exceed 17 % showing the reproducibility of the method. Concerning the phenolic compounds, some difficulties were encountered. Despite trying several extraction conditions, no protocol was found to be appropriate for the five phenolic compounds. Thus the compromise was made to have a low recovery for one compound, nonylphenol. As can be seen in Table 2, nonylphenol extraction led to recoveries of less than 20 %. Moreover, the presence of nonylphenol carboxylate and bisphenol A in WWTP effluents with concentrations 10 times higher than the spiking did not allow the recovery calculation in that case. Thus for WWTP investigation, NPEC and BPA were determined according to the recoveries obtained in spring water with the risk of having an underestimation.

Limits of detection

Two hundred ml of Evian spring water and WWTP effluent were spiked at a level close to the supposed limit of quantification (LOQ) (10 ng l^{-1}), then extracted and analyzed by LC-MS/MS. The limits of detection (LOD) and the limits of quantification were calculated for a signal-to-noise of 3 and 10, respectively. The analytical procedure was repeated 3 times and the mean value was calculated. Table 3 shows the LOD and the LOQ calculated for all compounds of interest.

Limits of detection were lower for steroid hormones, especially in WWTP effluents where the purification effect is

Table 2. Recoveries obtained according to the SPE procedures developed for steroid hormones, alkylphenols and bisphenol A.

Compound	R_1 (%)	RSD (n=3) (%)	R_2 (%)	RSD (n=3) (%)	R (%)	R_1 (%)	RSD (n=3) (%)	R_2 (%)	RSD (n=3) (%)	R (%)
E1	97.4	± 6.3	89.3	± 1.4	93.3	78.2	± 17.0	93.7	± 5.6	86.0
E2	105.6	± 8.3	87.8	± 2.9	96.7	69.3	± 12.6	92.3	± 4.6	80.8
EE2	79.6	± 2.4	92.6	± 1.2	86.1	73.4	± 8.0	87.7	± 6.0	80.5
OHE1	94.0	± 9.8	88.3	± 1.8	91.1	50.4	± 7.2	56.8	± 11.2	53.6
NP	13.7	± 17.3	20.8	± 12.7	17.2	15.3	± 10.9	10.6	± 7.1	12.9
OP	82.2	± 13.6	98.3	± 0.7	102.8	84.1	± 12.9	78.9	± 8.9	78.9
NPEC	107.4	± 4.5	66.4	± 13.4	74.3	-	-	-	-	-
OPEC	86.6	± 4.7	79.9	± 3.1	83.2	88.6	± 3.1	48.9	± 6.7	68.8
BPA	78.8	± 18.7	95.0	± 3.3	86.9	-	-	-	-	-

(-): not determined

Table 3. Limits of detection and limits of quantification for all compounds in spring water and WWTP effluents (n=3).

Compound	Spring water (Evian)		WWTP effluent	
	LOD (ng l^{-1})	LOQ (ng l^{-1})	LOD (ng l^{-1})	LOQ (ng l^{-1})
E1	0.02	0.06	0.02	0.06
E2	0.03	0.10	0.16	0.52
EE2	0.21	0.70	0.28	0.94
OHE1	0.03	0.08	0.05	0.18
NP	0.11	0.39	0.42	1.40
NPEC	0.34	1.15	2.29	7.65
OP	7.94	26.47	9.43	31.47
OPEC	0.31	1.05	7.46	24.86
BPA	0.03	0.11	10.17	33.89

obvious (LOD < 0.3 ng l^{-1}). In contrast, we observed significant differences for phenolic compounds between the results obtained with spring water and with WWTP effluents, showing the impact of natural organic matter on the analysis. No additional purification step was developed because of their lower estrogenicity. The limits of detection obtained without purification were sufficient to detect dangerous concentration levels of phenolic compounds.

Wastewater Treatment Plant Investigation

Concentrations of the target compounds were measured in a domestic French WWTP. The WWTP carries out classic processes such as primary treatments (screening), anaerobic and aerobic biological treatments, and a clarification step. Samples were collected and analyzed at each step of the treatment, except the primary treatment. Three series of measurements were made for hormones and two for phenolic compounds between March 2007 and June 2007. The concentration mean values and their standard deviation are given in Table 4.

EE2 and OPEC were not detected in raw water or other samples of the WWTP. For steroid hormones, the performances of the WWTP were shown to have overall degradation rates higher than 85.5 % (value obtained in the case of E1). Only E1 was detected in treated effluents at 5.4 ng l^{-1}. Removal after the activated sludge treatment ranged from 90 % to 100 % showing the high efficiency and importance of this step in a WWTP.

For alkylphenolic compounds and bisphenol A, the study revealed that the WWTP treatment was inefficient. Except for OPEC, all compounds presented overall removal rates less than 32 % of which 3 were negative. In fact NP, OP and NPEC are degradation by-products of alkylphenol polyethoxylate and significant negative removal rates were observed after the anaerobic treatment (up to - 700 % in the case of NPEC). As in the case of steroid hormones, activated sludge presented the best degradation rates but was not sufficient to give satisfactory performances. In treated effluent NPEC was measured at 962 ng l^{-1}, NP at 3.3 ng l^{-1}, OP at 24.6 ng l^{-1} and BPA at 162.3 ng l^{-1} showing that the WWTP is a source of estrogenic pollution and could disrupt endocrine regulations in exposed fishes.

Drinking Water Production

As surface water is used for drinking water production, it was necessary to observe the fate of endocrine disrupting compounds in a French Drinking Water Treatment Plant (DWTP). Raw water (surface water) and drinking water were collected and analyzed twice between April 2007 and June 2007. The mean values are given in Table 5.

Most endocrine disrupters of interest were not detected in the raw water of the DWTP. Two hormones and two phenolic compounds were detected in raw water with a maximum of 10.7 ng l^{-1} for BPA. Removal rates were higher than 82 % and some traces of NPEC (1.7 ng l^{-1}) and BPA (1.9 ng l^{-1}) were measured in drinking water. These values are assumed to be too low to have a negative effect on human health.

CONCLUSIONS

Environmental endocrine disruption raises many questions in the scientific community in term of human health. Hence determining endocrine disrupters, such as steroid estrogens and alkylphenolic compounds, in water represents a major analytical challenge. In this study, a liquid chromatographic tandem mass spectrometric method with high sensitivity and specificity was established for analyzing

Table 4. Concentration mean values of the target compounds (ng l^{-1}), standard deviation for steroid hormones and degradation rate at each step of a domestic WWTP.

Compound	Raw water (ng l^{-1})	After anaerobic treatment		After aerobic activated sludge		Treated effluent		Overall removal rate (%)
		Concentration (ng l^{-1})	Removal rate (%)	Concentration (ng l^{-1})	Removal rate (%)	Concentration (ng l^{-1})	Removal rate (%)	
E1	37.1±*5.0*	37.7±*13.7*	- 1.6	3.8±*0.9*	89.9	5.4±*0.7*	- 42.1	85.5
E2	7.5±*1.9*	4.9±*1.2*	34.7	< LOD	> 99.4	< LOD	-	> 97.3
EE2	< LOD	< LOD	-	< LOD	-	< LOD	-	-
OHE1	12.3±*4.2*	15.9±*4.9*	- 29.3	< LOD	> 99.7	< LOD	-	> 99.2
NP	< LOD	8.7	> 2075	5.1	41.4	3.4	33.3	< - 750
NPEC	137.5	1112.1	- 708.8	955.8	14.1	962.1	- 0.7	- 599.7
OP	16.3	13.5	17.2	14.0	- 3.7	24.6	- 75.7	- 50.9
OPEC	< LOD	< LOD	-	< LOD	-	< LOD	-	-
BPA	239.1	285.2	19.3	57.1	80.0	162.3	- 184.2	32.1

Table 5. Target compound concentrations (ng l^{-1}) in influent and treated water of a drinking water treatment plant.

Compound	Raw water (ng l^{-1})	Treated drinking water	
		Concentration (ng l^{-1})	Removal rate (%)
E1	0.55	< LOD	> 96.4
E2	< LOD	< LOD	-
EE2	< LOD	< LOD	-
OHE1	0.3	< LOD	> 90.0
NP	< LOD	< LOD	-
NPEC	9.6	1.7	81.8
OP	< LOD	< LOD	-
OPEC	< LOD	< LOD	-
BPA	10.7	1.9	82.2

four estrogens, four alkylphenols and bisphenol A in surface water and wastewater with SPE. The limits of detection of steroid hormones in 200 ml of WWTP effluents ranged between 0.02 ng l^{-1} and 0.21 ng l^{-1} thanks to an efficient purification step. For phenolic compounds the limits of detection ranged between 0.4 and 10.2 ng l^{-1}. Recoveries were higher than 50 % for all estrogens despite the matrix complexity. The results were not similar for alkylphenols and bisphenol A but sufficient to study the efficiency of a WWTP and a DWTP. The evolution of the target endocrine disrupters through a WWTP was observed and discussed. The efficiency of the treatment was shown for steroid hormones with overall removals of more than 85 %. However, the WWTP showed negative removal rates for alkylphenolic compounds and 33 % for bisphenol A. Thus, some EDCs such as estrone, octylphenol, nonylphenol, nonylphenol carboxylate and bisphenol A were still present in the effluent resulting in an estrogenic mixture being released into surface water. As the river is used for drinking water production, the presence of endocrine disrupters in drinking water was observed. Some traces of nonylphenol carboxylate and bisphenol A were detected but these cannot induce estrogenic properties in the drinking water.

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