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# Analysis of steroid hormones in effluents of wastewater treatment plants by liquid chromatoraphy–tandem mass spectrometry

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**Abstract**

This paper presents the development of an analytical procedure for the determination of two sexual steroid hormones: 17β-estradiol and estrone, and the synthetic contraceptive estrogen, 17α-ethynylestradiol in effluents of wastewater treatment plants. Samples are extracted via solid-phase extraction using $C_{18}$ cartridges. Extracts in ethyl acetate are then purified with a liquid–liquid separation with aqueous sodium chloride, then with a clean-up on a Florisil cartridge. Steroids are analyzed using an LC–MS–MS ion trap system. Internal quantification with the corresponding deuterated steroids leads to limits of quantification at 5 ng/l for estrone and 10 ng/l for estradiol and ethynylestradiol. In mineral spiked water, recoveries are 91% for 17β-estradiol, 97% for estrone and 87% for 17α-ethynylestradiol and RSDs are 15% for 17β-estradiol, 11% for estrone and 23% for 17α-ethynylestradiol.


*Keywords:* Water analysis; Steroids

## 1. Introduction

Endocrine disruption has become of increasing concern during the past decade. Compounds involved in these mechanisms may interfere with normal endocrine function of both wildlife and humans. Adverse effects due to endocrine disruptors include decreased sperm count, development of hormonally sensitive carcinomas (female breast cancers, testicular and prostate cancers) in humans. In wildlife, endocrine disruptors (EDCs) induce reproductive abnormalities, feminization of fish and decrease in the reproduction rate of birds [1].

A number of chemicals are involved in endocrine disruption. They include natural and synthetic steroid hormones, phytoestrogens, pesticides, surfactants and polychlorinated biphenyls [2,3]. The steroid hormones are of special concern due to their potency. The natural sex hormone estradiol and its metabolites (estrone and estriol) and the synthetic steroid ethynylestradiol are mainly excreted in the urine of mammals. They enter the environment through effluents of wastewater treatment plants (WWTPs) and wet-weather run-off. Their contributions to estrogenicity of WWTP effluents have already been identified [4–7].

Few methods are available to quantify steroid hormones in WWTP effluents. Most of them rely on a

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solid-phase extraction (SPE) followed by a derivatization step prior to detection by gas chromatography–mass spectrometry (GC–MS). Lee and Peart [8] developed such a method for the detection of estradiol, estriol, estrone and ethynylestradiol in sewage effluents. The detection limits were in the range 5–10 ng/l. A complex protocol combining high-performance liquid chromatography (HPLC) and GC–MS has been developed by Belfroid et al. [9]. It involved an SPE on disk. After extraction, the hormones were separated by HPLC and fractions containing the hormones were analyzed by GC–MS following derivatization. The limits of detection (LODs) ranged from 0.1 to 2.4 ng/l according to the substances and the matrix analyzed. A method for the determination of estrogens in river water and effluents using SPE and GC–negative chemical ionization (NCI) MS was described by Xiao et al. [7]. This method demonstrated LODs of 0.2 ng/l for estrone, 0.03 ng/l for estradiol and 0.05 ng/l for ethynylestradiol. Derivatization was time consuming and represented a critical phase of the sample preparation.

LC–MS–MS is a technology applicable to a wide range of molecules and matrices. It is generally preferred to GC–MS because of its sensitivity and specificity. Furthermore, no derivatization is required. Lagana et al. developed a method based on LC–MS–MS [10] which allowed for the quantification of 2 ng/l of estradiol and ethynylestradiol and 4 ng/l of estrone. Ionization was accomplished using atmospheric pressure chemical ionization (APCI). The detection limit achieved by Komori et al. with the same technology was 0.5 ng/l [11].

This communication describes a sensitive method for the determination of estradiol and its metabolites (estriol and estrone) and ethynylestradiol in WWTP effluents. Sample preparation was by SPE. Detection was performed using a LC–MS–MS with electrospray ionization (ESI). The mass spectrometer used in this study was equipped with a ion trap source.

## 2. Experimental

### 2.1. Chemicals

All solvents were analytical grade. Hexane, dichloromethane, acetone, ethyl acetate and water were purchased from J.T. Baker (Atlantic Labo, Eyssines, France). Methanol came from Merck (Fontenay sous Bois, France). Pure standards of estradiol (E2), deuterium-labeled [16,16,17-$^{2}$H]17β-estradiol (E2-$d_3$), estrone (E1), deuterium-labeled [2,4,16,16-$^{2}$H] estrone (E1-$d_4$), estriol (E3), ethynylestradiol (EE2) and mestranol were purchased as powders from Sigma–Aldrich (Saint Quentin Fallaviers, France). Deuterium-labeled [2,4,16,16-$^{2}$H]17α-ethynylestradiol (EE2-$d_4$) was from C/D/N Isotopes (CIL Cluzeau, Sainte Foy la Grande, France).

Glass microfiber filters (GF/D—2.7 μm pore size) were obtained from Whatman. $C_{18}$ SPE cartridges (1 g, 6 ml) were purchased from J.T. Baker. Supelclean LC-Florisil cartridges (1 g, 6 ml) were obtained from Supelco (Saint Quentin Fallaviers, France). A dewatering step was performed by passing the sample through internally prepared sodium sulfate cartridges. Sodium sulfate was obtained from Carlo Erba (Val de Rueil, France). The filtration tubes (6 ml) and polyethylene frits for 6-ml tubes were purchased from Supelco.

### 2.2. Preparation of standards

A stock standard solution containing all steroids at 100 mg/l was prepared in methanol. A working solution at 1 mg/l was prepared by dilution of the stock solution in methanol. All solutions were stored at −20 °C prior to use. Deuterium-labeled steroids were prepared individually in methanol at a concentration of 1 mg/l.

Calibration standards were prepared by spiking 1 l of mineral water (Evian) with the appropriate amounts of the working solution to achieve concentrations up to 50 ng/l. A fixed concentration of internal standards (deuterium-labeled steroids) at 20 ng/l was added to each standard solution prior to the extraction step.

### 2.3. Sample preparation

Effluents of some WWTPs were collected from January to August 2002. Samples were stored at 4 °C and extracted within 48 h to avoid any degradation of 17β-estradiol into estrone.

Prior to extraction, 20 ng/l (10 μl of 2 mg/l) of each internal standard were added to 1 l of sample for quantification. Samples were filtered through GF/D

glass fiber filters to avoid clogging of the cartridges. Steroids on the filters were sonicated twice with 5 ml of acetone for 15 min. The acetone extracts were collected and combined to the filtrate.

Sample extraction was performed with $C_{18}$ cartridges using a Visiprep system (Supelco). Prior to extraction, cartridges were conditioned sequentially with 5 ml of hexane, 5 ml of ethyl acetate, 5 ml of methanol and 10 ml of water. After loading 1 l of sample, the cartridge was dried under nitrogen during approximately 1 h. The steroids were desorbed with 6 ml of a mixture of ethyl acetate–methanol (5:1, v/v). The extracts were reduced to dryness under nitrogen and reconstituted in 2 ml of ethyl acetate.

A liquid–liquid separation was then carried out to eliminate hydrophilic interferences (such as humic substances). The ethyl acetate extracts were extracted twice with 1 ml of an aqueous 5% (w/v) sodium chloride solution. Ethyl acetate and sodium chloride were mixed for 1 min per extraction. The organic extracts were combined and dewatered by passing through anhydrous sodium sulfate cartridges. The cartridges were rinsed twice with 2 ml of ethyl acetate. The extracts were combined, the solvent was evaporated under nitrogen and reconstituted with hexane–methylene chloride (1:1, v/v) to a final volume of 0.2 ml.

Extracts were then purified on Florisil cartridges (Supelco). After conditioning with 10 ml of hexane–methylene chloride (1:1), extracts were placed at the surface of the cartridges. The elution was performed using 7 ml of methylene chloride–acetone (95:5, v/v). After evaporation to dryness under nitrogen, 50 μl of methanol was added. The sample was gently mixed before adding 150 μl of water prior to LC–MS–MS analysis.

Table 1
LC gradient conditions: A = water, B = acetonitrile

| | A (%) | B (%) |
|---|---|---|
| 0 min | 60 | 40 |
| 25 min | 20 | 80 |
| 30 min | 60 | 40 |
| Post time: 25 min | | |

### 2.4. LC–MS–MS analysis

The HPLC apparatus consisted of an Agilent 1100 autosampler, LC pumps and a column oven (Agilent, Massy, France). Separation was performed on a Hypersil BDS $C_{18}$ column (250×2.1 mm, 3 μm) with a guard column (2×2.1 mm, 3 μm) at a flow-rate of 0.2 ml/min. The injected volume was 50 μl. All solvents passed through a degassing module prior to entering the pressurized LC pumps. Table 1 presents the LC gradient conditions.

The detection was performed using an ion trap mass spectrometer LCQ from ThermoFinnigan. Two different interfaces were evaluated: ESI and APCI both in the positive and negative modes.

Table 2 presents the optimized MS–MS conditions in the negative ESI mode.

## 3. Results and discussion

For the chromatographic separation, mobile phases consisting of either water–methanol or water–acetonitrile were evaluated in terms of resolution, duration of the analysis and sensitivity. Based upon these criteria, the best overall results were achieved with water–acetonitrile eluents.

Table 2
Optimized MS–MS conditions for analysis of selected steroids in the negative ESI mode

| Compound | Normalized collision energy (%) | Activation (*Q*) | Parent ion (*m*/*z*) | Product ion (*m*/*z*) |
|---|---|---|---|---|
| Estradiol | 52 | 0.29 | 271.5 | 145.5 + 183.5 |
| Estradiol-$d_3$ | 52 | 0.28 | 274.5 | 145.5 + 185.5 |
| Ethynylestradiol | 49 | 0.25 | 295.5 | 145.5 + 185.5 |
| Ethynylestradiol-$d_4$ | 51 | 0.25 | 299.5 | 147.5 + 187.5 |
| Estrone | 49 | 0.28 | 269.5 | 145.5 + 183.5 |
| Estrone-$d_4$ | 50 | 0.28 | 273.5 | 147.5 + 187.5 |

ND: Not detected.

Table 3
Average recoveries of each sample preparation step for estriol, estradiol, ethynylestradiol and estrone

| | Recovery (%) | | | |
|---|---|---|---|---|
| | Estriol | Estradiol | Ethynyl-estradiol | Estrone |
| SPE | 94 | 84 | 84 | 98 |
| Liquid–liquid separation | 91 | 88 | 101 | 108 |
| Clean-up | 0 | 84 | 97 | 93 |
| Total procedure | 0 | 88 | 87 | 95 |

### 3.1. Sample preparation

Recoveries of each step of the sample preparation were evaluated individually on a standard solution spiked with steroids at 20 ng/l. The whole procedure was applied to the same standard solution. Recoveries corresponded to the ratio of the experimental concentrations to the theoretical concentration (20 ng/l). The results are presented in Table 3.

SPE, liquid–liquid separation and clean-up on Florisil consistently resulted in recoveries, respectively, in the ranges 84–98, 88–108 and 82–97% for all steroids other than estriol. Estriol did not elute from the cartridge after treatment with the methylene chloride–acetone (95:5) eluent. It was anticipated that elution with a more polar solvent mixture might desorb estriol as well as interfering compounds, thus adversely affecting analysis of E2, EE2 and E1. Estriol was excluded from further evaluation and the current procedure maintained.

### 3.2. Choice of the ionization mode

APCI and ESI interfaces were evaluated for the determination of the selected estrogens in both the positive or negative modes. Table 4 shows the intensity and signal/noise ratio (*S*/*N*) resulting from each configuration.

None of the steroids were detected with $ESI^+$. $APCI^-$ demonstrated a lower signal than $APCI^+$ or $ESI^-$. Sensitivities in $APCI^+$ and $ESI^-$ were in the same range, but the *S*/*N* ratios were greater with $ESI^-$, leading to a lower LOD. The $ESI^-$ interface was selected because it provided the best overall sensitivity for the steroids of interest.

### 3.3. Quantification

In mineral water, recoveries were generally in the range 70 to 90% for E2, EE2 and E1. In extracts of WWTP effluents, recoveries were lower than 60%. Matrix effects were more important at the beginning of the chromatogram than at the end as they decreased with an increase in retention time. They were higher for estradiol than for estrone. Thus it was necessary to perform quantification using internal standards (i.e., the corresponding deuterium labeled steroids).

WWTP effluents were spiked with 10 and 20 ng/l of steroids. Concentrations were determined using external and internal standard quantification procedures (Table 5). External standard quantification resulted in recoveries of 50–60% for estrone. Estradiol and ethynylestradiol concentrations appeared to be lower than the limit of quantification (LOQ) (10 ng/l) even

Table 4
Signal intensity and signal/noise ratio observed on a standard solution (12.5 ng injected on column)

| Compound | $APCI^+$ | | $APCI^-$ | | $ESI^-$ | |
|---|---|---|---|---|---|---|
| | Intensity ($\cdot 10^{-4}$) | *S*/*N* ratio | Intensity ($\cdot 10^{-4}$) | *S*/*N* ratio | Intensity ($\cdot 10^{-4}$) | *S*/*N* ratio |
| Estriol | 4.0 | 20 | 1.5 | 124 | 9.6 | 310 |
| Estradiol | 4.9 | 32 | 3.9 | 197 | 2.7 | 257 |
| Ethynylestradiol | 2.4 | 154 | 0.10 | 32 | 3.5 | 40 |
| Estrone | 19 | 40 | 0.85 | 411 | 260 | 566 |
| Mestranol | 5.2 | 250 | ND* | / | ND* | / |

ND: Not detected.

Table 5
Recoveries determined in WWTP effluent spiked at 10 and 20 ng/l of E2, EE2 and E1 with external and internal quantification

| Spike | External quantification | | | Internal quantification | | |
|---|---|---|---|---|---|---|
| (ng/l) | E2 | EE2 | E1 | E2 | EE2 | E1 |
| 10 | ND | ND | 60 | 100 | 94 | 99 |
| 20 | ND | ND | 50 | 95 | 75 | 75 |

Table 6
Calibration curve correlation coefficients ($r^2$), limits of detection (ng/l), recovery and repeatability for E2, EE2 and E1 in Evian mineral water

| | $r^2$ | LOD (ng/l) (quantity injected) | Recovery (%) ($n=5$) | RSD (%) ($n=5$) |
|---|---|---|---|---|
| Estradiol | 0.9989 | 2 (500 pg on column) | 91 | 15 |
| Ethynylestradiol | 0.9972 | 2 (500 pg on column) | 87 | 23 |
| Estrone | 0.9979 | 1 (250 pg on column) | 97 | 11 |

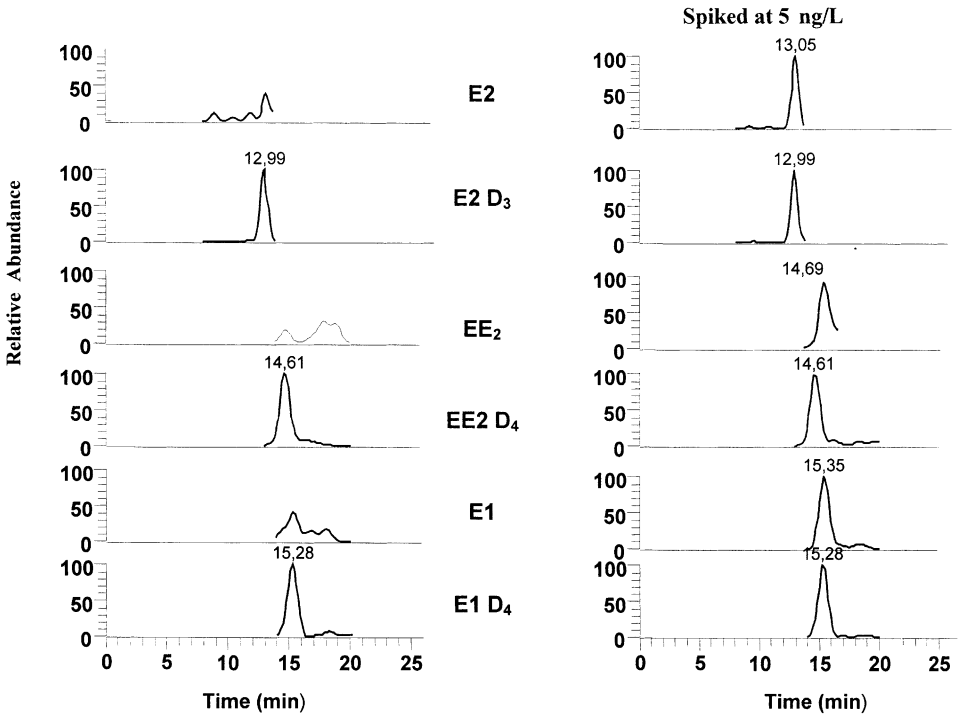


Fig. 1. LC–MS–MS chromatogram of a WWTP effluent and a WWTP effluent spiked at 5 ng/l in E2, EE2 and E1 and 20 ng/l in E2-$d_3$, EE2-$d_4$ and E1-$d_4$.

Table 7
Concentrations of estradiol, ethynylestradiol and estrone in three WWTP effluents (ng/l)

| Sample | Estradiol | Ethynylestradiol | Estrone |
|---|---|---|---|
| 1 | <10 | <10 | <5 (2) |
| 2 | <10 | <10 | <5 (1) |
| 3 | <10 | <10 | 5 |

Figures in parentheses indicate concentrations between LOD and limit of quantitation.

at a spiking level of 20 ng/l. With internal standard quantification recoveries ranged from 75 to 100%.

### 3.4. Method performance

The performance of the method was evaluated by the determination of the linearity, sensitivity and repeatability of the method.

Calibration curves were established using a least-square linear regression from the injection of standard solutions at concentrations from 5 to 50 ng/l for estrone and 10 to 50 ng/l for estradiol and ethynylestradiol. The LODs were determined from the injection of a standard solution at 10 ng/l of each steroid. The LOD was defined by the concentration for which the signal-to-noise ratio is at least three. The repeatability and accuracy of the overall protocol were evaluated from the injection of five replicates of Evian mineral water spiked at 20 ng/l of all steroids. The quantification was performed using internal standards.

Table 6 presents the correlation coefficients ($r^2$) for each compound. For all estrogens, the linearity was satisfactory since $r^2$ was higher than 0.99. The LODs were 2 ng/l for estradiol and ethynylestradiol and 1 ng/l for estrone. Recoveries were higher than 80% for all compounds. Ethynylestradiol demonstrated the highest RSD at 23%. Nevertheless, the repeatability of the method was adequate for all estrogens.

### 3.5. WWTP effluents

This method was successfully applied to effluents of WWTPs (Table 7). Fig. 1 shows a typical chromatogram from an effluent spiked at 10 ng/l with estradiol, ethynylestradiol and estriol. In the WWTP effluents studied, concentrations were always lower than 10 ng/l for estradiol and ethynylestradiol. Estrone was detected in all samples at concentrations from 1 to 5 ng/l.

## 4. Conclusion

This communication summarized the development and the validation of a method which is applicable to WWTP effluents with LOQs of 5 ng/l for estrone and 10 ng/l for estradiol and ethynylestradiol. Recoveries were higher than 80% and the repeatability was less than 25%. Future investigations will compare the results obtained from the LCQ with results from other LC–MS–MS systems in order to reach LOQs at the sub-ng/l level.

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