# Detection of Estrogens from Aquatic Environments by Gas Chromatographic Method

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*The presence of estrogens in the environment and their effects especially on reproductive health on aquatic animals and on humans has become a subject of growing concern worldwide. This paper describes the preliminary data and tests performed in order to obtain and optimize a new gas chromatographic method for the identification of the most common found estrogen in the environment, 17α-ethinylestradiol. For this purpose, a gas chromatograph equipped with an electron capture detector and a chromatographic column DB-5MS has been used and the specific parameters have been optimized. The developed analytical method was then tested on two types of water (waste water and surface water). The processing of samples followed several steps, involving liquid-liquid extraction, concentration, purification and derivatization. The linearity range of the method has been established between 0.025 - 0.100 μg/L. The recovery for 17á-ethinylestradiol fortified water samples reached 85.30 %.*

*Keywords: aquatic environment, estrogens, GC-ECD method.*

The endocrine system disruption is a widely discussed topic worldwide, leading to numerous studies in this area [1, 2]. A large number of chemicals both of natural and anthropogenic origin from the environment have been identified to affect the normal functions of the endocrine system, despite their low concentrations [3]. These chemical substances have been defined as *endocrine disruptors* (ECD) [4]. They have the ability of imitating normal functions of natural steroid hormones, or antagonize their actions, leading to alteration of their biosynthesis or metabolism, or even alteration of entire hormone populations [5].

The most important sources of endocrine disruptors in the environment are the wastewater treatment plants [6]. Surface water quality is very important [7, 8] the municipal wastewaters polluted with heavy metals, pharmaceutical residues and other toxic pollutants are discharged without proper treatment into surface water being able to reduce biodiversity [9, 10]. Hormones are one of the most potent endocrine disruptors. Some of them have limits in their use and lacking a specific legislation [11].

Endocrine active drugs including substances used to improve fertility as well as contraceptives and hormone therapy have experienced a significant development [12]. The negative effects of estrogens on the environment altering the development of reproductive and nervous systems of living beings have been discussed extensively over many years, and numerous laboratory experiments have been performed [13-16]. From the endocrine point of view, the endocrine hormones with interest to the aquatic environment are the estrogens (17β-estradiol, estrone, estriol, 17α-ethinylestradiol (EE2) and mestranol). The steroid estrogens are characterized by an aromatic ring containing a hydroxyl group in position C-3. For ethinylestradiol, the presence of an ethinyl group at C-17 offers to this hormone a higher resistance to degradation compared to the natural hormone [17]. In surface waters, natural and synthetic estrogens are present in extremely low concentrations, making the development of analytical methods a difficult process [18].

This paper presents the experiments performed in order to develop a new chromatographic method for the separation and identification of the most common synthetic estrogen found in the aquatic environment, 17α-ethinylestradiol and for testing on water samples.

## Experimental part

### *Prepared reagents and standards*

The standard for synthetic hormone 17á-ethinylestradiol, Vetranal 99.4% (EE2) and the trimethylamine hydrochloride, 98% (TMA) were obtained from Sigma Chemical Co. Pentafluoropropionic anhydride, 98.6% (PFPA) was obtained from Fluka. Solid-phase extraction (SPE) cartridges amino propyl, cyano propyl and silica were purchased from Agilent Technologies, Inc. Ultrapure water was obtained through a Micropure Ultrapure water system (TKA, Germany). Other chemicals and solvents used were of analytical purity.

All the glassware used has been washed with detergent solution, after which it was kept in an oxidizing mixture for 24 hours, then rinsed with ultrapure water, methanol, acetone and methylene chloride and placed in an oven at a temperature of 400°C to remove any trace of organic compounds. Only the volumetric glassware was dried at room temperature and away from dust.

Primary stock solution (10 mg/L) was prepared by dissolving 0.0010 g of 17α-ethinylestradiol in 100 mL of ethanol. Working solutions were daily prepared in various concentrations by appropriate dilution of the stock solution in ethanol. All solutions were stored at 4 °C in the dark. The solutions of trimethylamine (1 M) in toluene and potassium phosphate buffer (0.1 M) were prepared according to the procedure presented by Evans and collaborators [19].

Figure 1 schematically represents the flow diagram with the needed steps for the preparation of the standards used to establish the operational parameters of the gas

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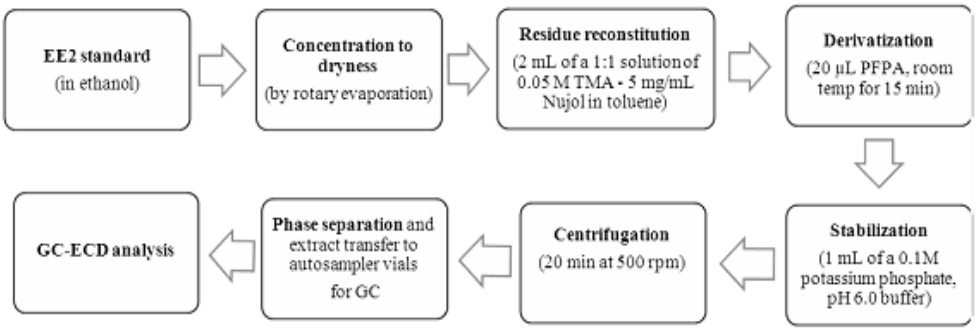


Fig. 1. Analytical flow diagram used for the EE2 derivatization to PFP-EE2

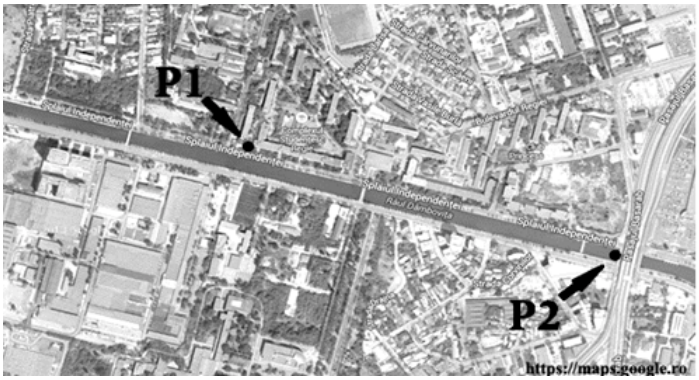


Fig. 2. Locations of the water sampling points from Dambovita River – Bucharest

chromatographic (GC) method of analysis and for the plotting of the calibration curve.

*Environmental sample collection, preparation and preservation*

There were two types of water samples collected from Bucharest, denoted P1 - wastewater samples collected from the final discharge point of the Regie Student Campus prior to discharge into Dambovita River, and P2 - surface water samples collected directly from Dambovita River, near Grozavesti Bridge as shown in figure 2.

## Experimental part

In order to avoid the addition of preservatives reagents, the water samples were directly transported to the laboratory immediately after collecting and subjected to the extraction procedure. Water samples were not filtered before extraction due to the fact that the analyte of interest, the synthetic hormone EE2, has a high value (4.15) of octanol-water partition coefficient. This property indicates the presence of high affinity for suspended matter and the filtering can produce significant losses of this compound [17, 20]. In parallel to the collected water samples, the control samples, the ultrapure water and the fortified water samples were processed following the same procedure.

*Extraction procedure*

After the addition of sodium thiosulphate (160 mg) to eliminate interference caused by the presence of chloride, the water samples (2 L), were extracted with dichloromethane (100 mL) for 2 min [21]. To remove any traces of water, the extract was transferred to a conical glass where anhydrous sodium sulfate was added. Extraction and extract drying procedure were repeated 3 times. After drying, concentration of the extract to dryness followed, using a rotary evaporator. The residue was reconstituted in 10 mL of 0.5 % isopropyl alcohol/hexane (I/H) and then purified using three types of cartridges.

*Clean-up and derivatization*

All three types of cartridges were initially preconditioned by passing of 10 mL solution of I/H (5 %) and then re-equilibrated with 30 mL of hexane. In the first SPE phase, CN cartridges were used. After the passing of the sample through the cartridge, it was washed with 35 mL of I/H (5 %) solution before the application of the extract. This extract was then concentrated to dryness, and the residue was reconstituted in 2 mL I/H (1 %). In the second SPE phase, silica cartridges have been used. After passing of the extract, the cartridge was washed with 10 mL of I/H (5 %). The eluted extract was concentrated to dryness using the rotary evaporator and the residue was reconstituted in 2 mL I/H (1 %). In the third SPE phase, amino propyl cartridges have been used. After the passing of the extract, the cartridge was washed with 10 mL of I/H (2 %), followed by an additional 5 mL of I/H (8 %). These extracts were dried and reconstituted with 2 mL of 1:1 solution of TMA-Nujol in toluene. The reconstituted extract was transferred with a Pasteur pipette to 10 mL glass-lined screw-capped tubes. After mixing, the samples were derivatized by the addition of 20µL of PFPA. They were left at room temperature for 15 min, shaking them occasionally. The derivatization reaction is carried out in this way, leading to the compound of interest EE2 pentafluoropropionyl (PFP-EE2), using TMA as catalyst. To complete the reaction, 1 mL of 0.1M potassium phosphate (*p*H 6.0) buffer was added, followed by centrifugation at 500 rpm for 20 min. The hydrolysis of the compound formed by derivatization was prevented by adding an additional 1 mL of buffer, followed by centrifugation. After the last step of removing the excess of unprotonated TMA and phase separation, the organic phase was carefully transferred into auto sampler vials for GC with PFPEs-lined caps and then analyzed.

*Gas chromatographic analysis*

GC analyses were performed on a CP 3800 gas chromatograph (Varian Inc., USA) using a capillary column (DB-5; 30 m x 0.22 mm x 0.25 µm) and equipped with an electron-capture detector (ECD). Before analyzing the collected water samples, the optimal working parameters for the gas chromatograph were determined and the linear range of the method was established.

## Results and discussions

*Method development and optimization*

The sensitivity of the chromatographic method of analysis depends on the following operational parameters: the flow rate of the mobile phase, the gas flow on the detector, injector, the detector temperature and the temperature program of the column oven.

*Optimization of the flow rate of the mobile phase*

An important parameter influencing both the intensity of the signal generated by the analyte concentration in the sample and the retention time of the compound is the flow rate of the mobile phase through the chromatographic column. To optimize this parameter, standard solution of 1 mg/L EE2 was injected, keeping all parameters of the method constant and varying the flow of the mobile phase,

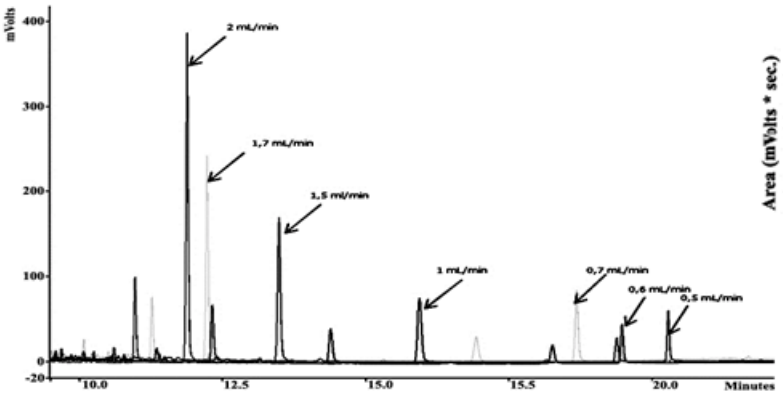


Fig. 3. Chromatograms obtained at different nitrogen flow values

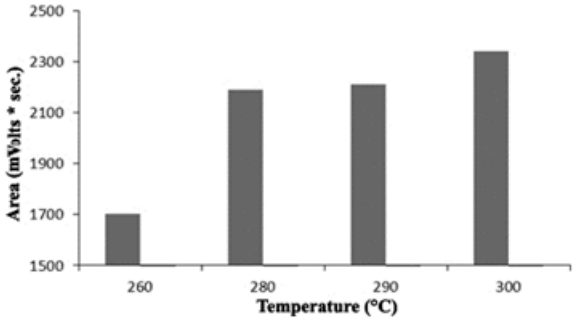


Fig. 4. Variation of the detector signal according to the detector temperature

| | |
|---|---|
| *Carrier gas* | Nitrogen: 2 mL/min |
| *Make-up gas* | Hydrogen: 5 mL/min |
| *Temperature program* | Initial temperature 90 $^0$C, held for 1 min; increased to 245 $^0$C at a rate of 20 $^0$C/min held for 10 min, and finally to 300 $^0$C, at a rate of 20 $^0$C/min and held for 10 min. (26.25 min analysis time) |
| *Injector temperature* | 290 $^0$C |
| *Detector temperature* | 300 $^0$C |
| *Solvent* | toluene |

**Table 1**
OPERATING CONDITIONS OF THE GC-ECD

monitoring the detector response (area and intensity of the analytical signal). Optimum flow rate of the mobile phase was set at 2 mL/min (fig.3).

*Optimization of the injector temperature and of the splitting rate*

In order to achieve an optimal temperature of the injector and the necessary split rate, repeated injections have been performed using the same standard solution of 1 mg/L ethinylestradiol, while keeping all the theoretical parameters of the method constant and varying the temperature of the injector (285, 290, 295 $^0$C) and the split rate (30, 20, 15 and 5). The optimum temperature of the injector was obtained at 290 $^0$C, in which case the greatest area has been obtained for the target analyte, and the maximum signal strength has been obtained for a split ratio of 1:5.

*Optimization of the temperature and of the gas flow on detector*

The optimum temperature of the column was set at 300 $^0$C and the optimal gas flow of the detector was set at 5 mL/min, according to the criteria for maximum analytical signal strength and separation (fig. 4). Different temperature programs were investigated for GC oven. At the end of this investigation, the best temperature program was selected for a good resolution.

The optimal operating conditions of the new analytical method are shown in table 1. The EE2 retention time was of approximately 9.69 min, with a good formation of the peaks in these conditions. Preliminary precision and linearity studies carried out during the development of the method have shown that the injection volume of 2µL was replicable and the signal was significant at the selected analyte concentrations.

*Linearity*

Standard solutions were injected into the gas chromatograph in ascending order regarding concentrations using triplicate injections. Dependence of peak area was plotted according to the concentration and linear regression was performed. A very good linearity of the method was obtained from the analytical signal by the concentration range between 0.025 - 0.100 µg/L (R=0.9975) with a detection limit of 0.005 µg/L.

The developed analytical method was applied to water samples collected from the selected locations. Figure 5.a shows the chromatograms obtained from the analysis of a 2 L water sample collected from Dambovita River denoted P2, of a water sample fortified with 0.05 mg/L EE2 denoted P2F and of a standard sample of 0.05 mg/L EE2. Figure 5.b shows the chromatograms obtained for the blank ultrapure water and for the fortified samples with two different levels

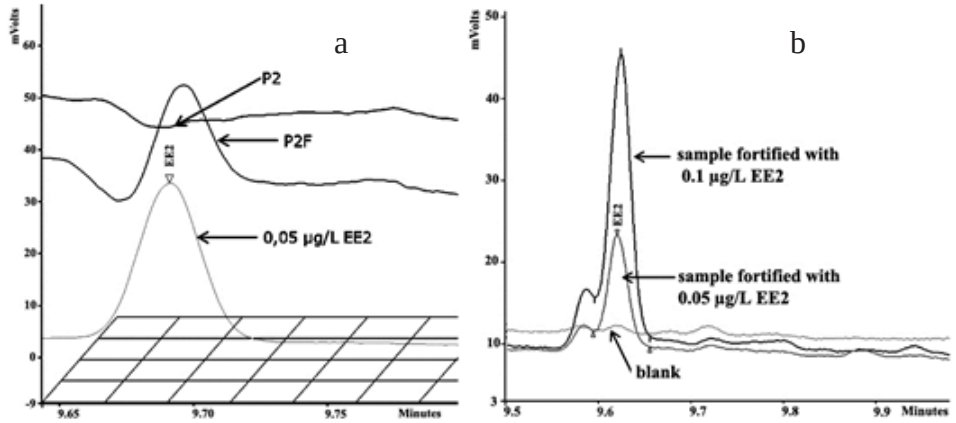


Fig. 5. Overlay of GC-ECD chromatograms of surface water samples (P2), fortified water samples with 0.05 µg/L (P2F) and 0.05 µg/L EE2 standard (a); chromatograms of ultrapure water samples and ultrapure water samples with fortified 0.05µg/L and 0.10 µg/L EE2 (b)

of concentrations (0.05 and 0.1vg/L of EE2). The content of 17α-ethinylestradiol in the collected samples ranged below the limit of detection for this method and the recovery of EE2 for the fortified samples reached 85.30 %.

## Conclusions

A gas chromatographic method with electron capture detection for the determination of the most important synthetic estrogen (17α-ethinylestradiol) present in the aquatic environment has been developed and a procedure for water samples processing for the determination of endocrine disruptors has been established.

The injector and detector temperatures were set to 290 and 300 $^0$C respectively. The temperature program of the GC oven with a run time of 26.25 min was established. All injections were performed using a volume of 2μL. The 17α-ethinylestradiol content from the analyzed samples ranged below the limit of detection for this method. The newly developed analysis method may be used for the synthetic estrogen detection in wastewater and surface water samples.

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