Detection of Estrogen Hormones in Danube River and Tributaries Using Liquid Chromatography-Mass Spectrometry

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A sensitive and selective LC-MS/MS method with negative electrospray ionization was developed to detect six estrogen hormones from Danube River and three major tributaries (Jiu, Olt and Arges). The method separates the estrogen hormones in 25 min using a C18 end-capped column (150 x 2.1 mm, 3.5 μm) kept at 20ºC and a mobile phase made of aq. 0.01% NH_4OH and MeOH in the ratio 47.5/52.5 (v/v). Elution program allowed baseline separation of the hormones and consisted of an isocratic step (10 min) followed by a slow gradient (2.5%/min) applied in 4 min. Collision energy, fragmentor and capillary voltages were optimized to increase MS sensitivity. Optimization of LC-MS parameters generated extremely low instrumental LOQ values between 0.06 and 1.1 μg/L. MS detector response was linear in the range 1 ÷ 200 μg/L with R^2 > 0.99 for all hormones. SPE using Oasis HLB cartridges was employed to concentrate target analytes from water samples. Intra-day and inter-day precision (RSD%) was situated between 5.7 ÷ 8.9% and 8.4 ÷ 12.6%, respectively. Hormone recovery after SPE was good due to internal standard correction with values between 78 ÷ 110%. Overall method LOQs were situated between 0.2 and 3.3 ng/L. Only Estrone was found in Danube and its tributaries at levels from LOQ to 3.8 ng/L. Higher levels of Estrone were detected in tributaries when compared to Danube. Also locations downstream major cities showed higher levels than upstream ones.

Keywords: LC-MS/MS, estrogen hormones, Danube River and tributaries, solid-phase extraction

Estrogen hormones are female sex hormones responsible for growth and reproduction in human body, being present in both female and male genders with a plus for the former. Also, estrogen hormones are considered endocrine disrupting chemicals (EDCs) along with some pharmaceuticals and chemicals which are all present in the environment [1-4]. These compounds have recently come to the attention of researchers all over the world due to their properties that affect the reproduction and development of living organisms [5]. Among these compounds, hormones have a high endocrine disrupting potential and thus they are dangerous to humans even at very low concentrations (ng/L). Increasing amounts of EDCs including natural or synthetic hormones are introduced into the environment through untreated wastewaters or direct excretion. This is the main reason for which an increasing number of studies that detect hormones in the environment have been reported [6, 7]. The low levels of estrogen hormones present in surface water require very sensitive methods for their accurate detection. LC-MS/MS with ESI ionization coupled with SPE extraction is the method of choice when analyzing hormones in waste or surface water due to its versatility, sensitivity (ng/L), selectivity and reduced analysis time [5, 8].

The aim of the present study was to develop, optimize and validate a sensitive, selective, and accurate SPE-LC-MS/MS method able to determine six estrogen hormones (Equilin, Estrone, α-Estradiol, β-Estradiol, α-Ethinylestradiol and Estriole) from surface water at trace level concentration (ng/L). The method was applied to establish the occurrence of these estrogen hormones along the Romanian part of the Danube River and three of its main tributaries: Jiu, Olt and Arges.

Experimental part

Reagents and chemicals. HPLC grade acetonitrile and methanol were acquired from Merck. HCOOH (p.a.) and NH_4OH 25% (p.a.) were obtained from Sigma-Aldrich. HPLC grade water was obtained in-house using a MilliQ instrument. High purity reference standards of hormones: Equilin, Estrone, 17α-Estradiol, 17β-Estradiol, 17α-Ethinylestradiol, and Estriole were acquired from Sigma-Aldrich. Isotopically labelled hormones: 17β-Estradiol-16,16,17-d3 (βE2-d_3) and $^{13}C_2$-Ethinylestradiol ($^{13}C_2$-EE2) were used as surrogate internal standard and respectively injection internal standard for the quantitation of the six targeted estrogen hormones. βE2-d_3 was obtained from TRC Inc. (Toronto, Ontario, Canada), while $^{13}C_2$-EE2 was acquired from CIL (Andover, MA, USA). Oasis HLB (500 mg/6 mL) cartridges were aquired from Waters (Milford, Massachusetts, USA). Nine calibration solutions in the range 1 - 200 μg/L were obtained by successive dilutions from a 2 βg/mL mixed hormones stock solution.

LC-MS instrumentation and conditions

Experiments were performed using an Agilent 1260 series LC system (Waldbronn, Germany) consisting of: binary pump, autosampler, and thermostatted column compartment coupled with an Agilent 6410B triple-quadrupole mass spectrometer with electrospray ionization source (ESI). Chromatographic runs were carried out on a Zorbax Eclipse Plus C18 (150 x 2.1 mm, 3.5 μm) column Agilent Technologies which was kept at 20°C. All experiments were performed in gradient elution conditions at a flow-rate of 0.25 mL/min. Mobile phase composition was a mixture of aq. 0.01% NH_4OH and MeOH. The employed gradient elution program is given in table 1. To enhance method sensitivity, the sample injection volume

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Time (min)	Aq 0.01% NH_4OH (%)	MeOH (%)
0	47.5	52.5
10	47.5	52.5
14	37.5	62.5
20	47.5	52.5

Table 1
GRADIENT ELUTION PROGRAM USED TO SEPARATE THE SIX ESTROGEN HORMONES.

Compound	Retention time (min)	log Kow	MRM transitions	Fragmentor voltage (V)	Collision energy (V)	Dwell time (msec)
Estriole	5.2	2.67	287.1 → 171 287.1 → 145	180	36 43	300
$^{13}C_2$-EE2	21.6	-	297.1 → 145 297.1 → 143	200	43 43	120
Etinylestradiol	21.6	3.90	295.1 → 159 295.1 → 145	200	43 43	120
βE2-d_3	20.9	-	274.1 → 185 274.1 → 145	200	50 50	120
β-Estradiol	21.0	3.75	271.2 → 183 271.2 → 145	180	40 43	120
α-Estradiol	23.2	3.75	271.2 → 183 271.2 → 145	180	40 43	120
Estrone	20.4	4.31	269.1 → 145 269.1 → 143	180	43 43	120
Equilin	19.2	3.90	267.1 → 265.1 267.1 → 143	180	20 38	120

Table 2
RETENTION TIME, MRM TRANSITIONS, FRAGMENTOR VOLTAGE, COLLISION ENERGIES AND LOG K_{OW} VALUES [9] FOR THE ESTROGEN HORMONES

was increased up to 50 µL. Detection was achieved using Multiple Reaction Monitoring (MRM) acquisition mode. Retention time, MRM transitions fragmentor voltages, collision energies and other MS parameters are given in table 2. ESI ionization source was operated in negative mode with 320°C as the drying gas temperature, 10 L/min drying gas flow, 50 psi nebulizer pressure and 6000 V capillary voltage. Two MRM transitions were used, one for quantitation (quantifier) and another for analyte confirmation (qualifier) (table 2).

Sampling sites and sample collection

Water samples were collected in October 2015 from 10 locations along the Romanian part of the Danube River and 2 locations from each of the 3 tributaries (fig. 1), as follows: (a) Jiu river - upstream and downstream Craiova city; (b) Olt river - downstream Slatina and a site closed to its confluence with Danube; (c) Arges river - downstream Pitesti city and a site closed to its confluence with Danube, according to figure 1. Water samples were collected in 1L glass bottles, previously rinsed in the laboratory with methanol and with the sample on location. After collection, the samples were kept at 4°C until analysis. Sampling point geographical location is given in table 3.

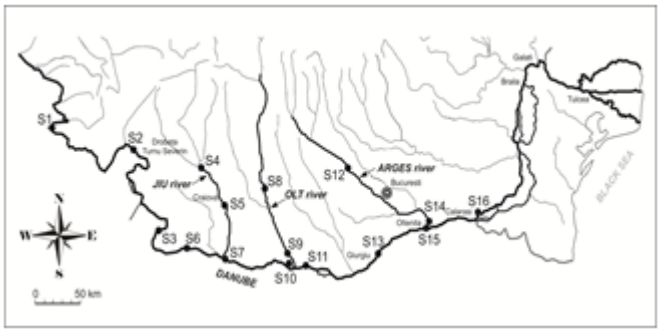

Fig. 1. Geographic position of sampling sites of the collected surface water samples

Results and discussions

LC separation optimization. The LC method was developed to obtain high efficient analyte peaks and good separation between them in the shortest possible time. Estrogen hormones are compounds with moderate to high hydrophobicity (log Kow = 2.67 ÷ 4.31) [9]. Hence, the chosen mobile phase composition was rich in organic solvent. A short retention study was done using both ACN (35 ÷ 50%) and MeOH (45 ÷ 55%) as organic modifier. The best separation in the shortest run-time was obtained when using 52.5% MeOH. The isocratic step of the elution program was used to increase retention for Estriole the most polar hormone (log Kow = 2.67) and allow for the separation of the other five. Several gradient slopes were tested to obtain the final separation. Thus, 2.5, 5.0 and 7.5%/min MeOH increase were investigated. The slowest gradient slope of 2.5%/min MeOH increase was sufficient to allow baseline separation of Equilin, Estrone, β-Estradiol, Ethinylestradiol, α-Estradiol. Concerning the elution order

Sampling point	River	GPS coordinates
S1 - Bazias	Danube	44°47'32.61"N, 21°23'20.07"E
S2 - Gura Vaii, upstream Drobeta Turnu Magurele	Danube	44°40'7.40"N, 22°33'10.74"E
S3 - Calafat	Danube	43°57'50.94"N, 22°54'15.72"E
S4 - Filiasi, upstream Craiova city	Jiu	44°34'8.32"N, 23°27'18.14"E
S5 - Podari, downstream Craiova city	Jiu	44°15'18.48"N, 23°47'25.08"E
S6 - Rast, upstream confluence with Jiu river	Danube	43°51'24.84"N, 23°17'18.79"E
S7 - Bechet, downstream confluence with Jiu river	Danube	43°45'11.32"N, 23°56'30.69"E
S8 - Downstream Slatina city	Olt	44°23'29.63"N, 24°21'4.84"E
S9 - Izbiceni, upstream confluence with Danube	Olt	43°48'41.71"N, 24°42'27.71"E
S10 - Islaz, upstream confluence with Olt river	Danube	43°42'22.72"N, 24°44'4.01"E
S11 - Drobeta, downstream confluence with Olt river	Danube	43°43'2.69"N, 24°48'56.65"E
S12 - Downstream Pitesti city	Arges	44°28'45.25"N, 25°40'47.87"E
S13 - Giurgiu, upstream confluence with Arges river	Danube	43°52'37.50"N, 25°58'49.92"E
S14 - upstream confluence with Danube river	Arges	44° 6'38.09"N, 26°38'15.33"E
S15 - Oltenita, downstream confluence with Arges river	Danube	44° 3'51.89"N, 26°38'45.49"E
S16 - Calarasi	Danube	44° 8'15.69"N, 27°20'8.26"E

Table 3
GEOGRAPHIC LOCATION WITH GPS COORDINATES AND DESCRIPTION OF SAMPLING SITES

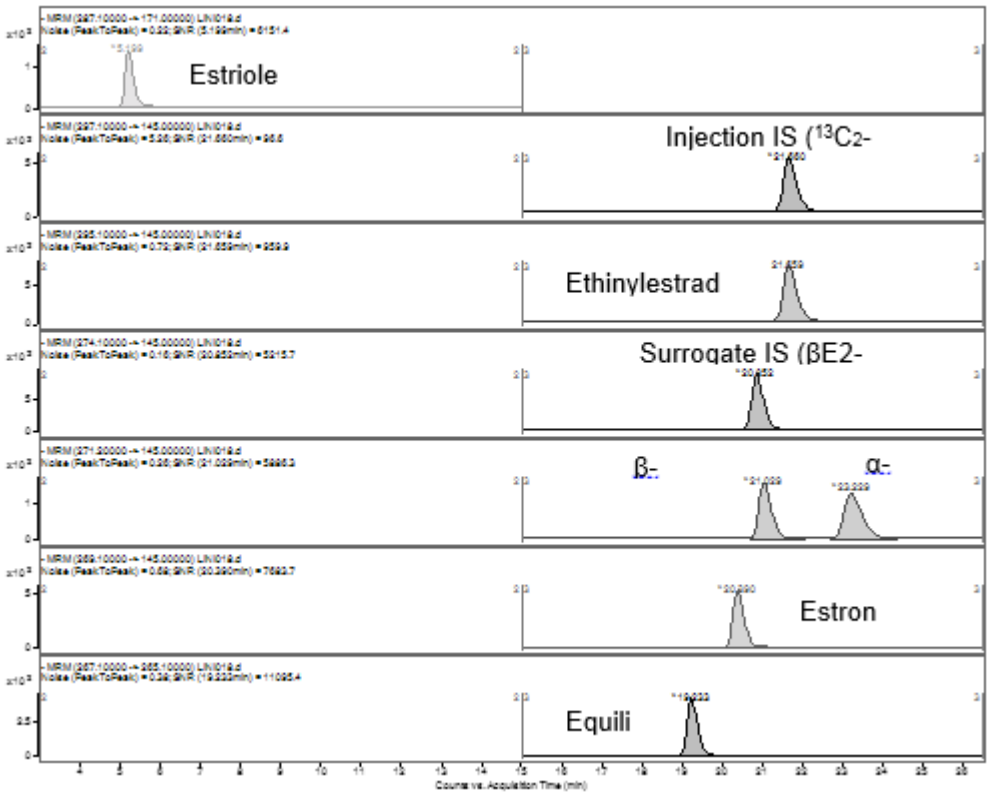

Fig. 2. MRM overlaid extracted ion chromatograms of a mixed 100 µg/L solution of the six hormones and the 2 internal standards (surrogate and injection internal standards)

of the hormones (table 2), it can be observed that it is not according to the log Kow value increase as it would be expected [10]; this is due to the dissociation of the OH groups in the chosen basic mobile phase (*p*H ~ 9.5) and to mobile phase solubility of each compound. Mobile phase flow-rate was varied between 0.2, 0.25 and 0.3 mL/min. The final chosen value was 0.25 mL/min because it allowed both fast separation and good MS sensitivity of the analytes, keeping in mind that ESI ionization is more efficient at lower mobile phase flow-rates.

MS detection optimization

MS detection parameters were optimized to obtain maximum sensitivity for the hormones quantitation. Analytes ionization in the MS source was tested in both polarity modes (±). Due to the acidic phenol groups in their molecule, negative ESI ionization proved to be much more sensitive than positive one for these compounds. Also, one of the most important factors affecting analyte ionization in the ESI source of the MS detector is the nature of mobile phase. So, aqueous HCOOH (0.1-0.2%), $HCOONH_4$ (5-20 mM), CH_3COONH_4 (5-20 mM) / NH_4OH buffer, NH_4OH (0.005-0.01%) were tried as mobile phase additives, whereas for the organic component of the mobile phase ACN and MeOH were used. HCOOH and NH_4 salts were disregarded due to poor ionization efficiency. Instead, aq. NH_4OH (0.01%) showed both good sensitivity and peak shape for the six hormones. Among the two organic solvents, ACN generated poorer sensitivity by increased background noise when compared to MeOH and thus the latter was chosen. Other ionization source parameters were also modified in a wide range to obtain highest ionization efficiency for the six hormones. Capillary voltage (3000 ÷ 6000V), drying gas temperature (300 ÷ 350°C) and drying gas flow (8 ÷ 12 L/min) were optimized with respect to hormone MS response. The final chosen values were: 6000V capillary voltage, 320°C drying gas temperature and 10 L/min drying gas flow. Collision energy (CE) applied in the collision cell (Q2) to the precursor ions to dissociate them and obtain product ions was varied between 10 and 60V with a 5V increment. CE between 35 - 50V generated highest dissociation yield of the precursor ions for the six hormones. A finer optimization of the CE was performed in this range using a smaller step (1V). Finally, CE showing the highest S/N ratio values for each analyte was chosen for the MRM transitions (table 2). After the entire procedure of MS optimization, the obtained instrumental quantitation limits were lower than 0.5 µg/L, except for á-Estradiol with a higher value of 1.1 µg/L (table 3).

Automated SPE optimization

All parameters of the automated SPE extractor were varied to determine the most efficient procedure to concentrate target analytes from surface water. Elution solvent (MeOH, ACN) and volume (5, 10 and 15 mL), sample loading flow-rate (5, 10 and 15 mL/min) and drying time (20, 30, 40 min) were modified to determine the values for which the maximum recovery of the hormones is achieved. The optimized extraction procedure is given in the following: SPE cartridge conditioning was done with 2 x 5 mL MeOH and then 2 x 5 mL H_2O. Water samples (500 mL) were filtered (0.45 µm cellulose) and spiked with 1 mL of 100 µg/L surrogate internal standard solution (βE2-d_3). Samples were loaded on the SPE cartridge (Oasis HLB - 500 mg/6 mL) at a constant flow-rate of 10 mL/min. After adsorption, the cartridges were air-dried for 40 min and then the analytes are desorbed with 2 x 5 mL MeOH. Extracts are then evaporated to dryness under a gentle N_2 stream (45°C). 100 µL of 1 µg/mL injection internal standard ($^{13}C_2$-EE2) are added to the dried samples and then the volume is brought to 1 mL with MeOH:H_2O mixture (50/50). Automated SPE provided 500 fold concentration of the water samples, which allowed determination of the six estrogen hormones down to few ng/L (LOQs between 0.2 – 3.3 ng/L).

SPE-LC-MS/MS method validation

To account for its performance, the developed SPE-LC-MS/MS method was validated with respect to specificity, linearity, precision, accuracy and limit of quantitation. MS detector response proved to be linear in the range 1 ÷ 200 µg/L with high determination coefficients (R^2 > 0.99).

Table 4

DETERMINATION COEFFICIENTS (R^2), INTRA-DAY AND INTER-DAY PRECISION, ANALYTE RECOVERY (ACCURACY), INSTRUMENTAL QUANTITATION LIMIT (IQL) AND METHOD QUANTITATION LIMIT (LOQ)

Analyte	R^2	Precision (%)		Overall method recovery (% ± RSD%)	IQL (µg/L)	LOQ (ng/L)
		Intra-day	Inter-day			
Estriole	0.9987	8.9	12.6	78 ± 8	0.21	0.6
Equilin	0.9941	5.7	8.4	86 ± 6	0.06	0.2
Estrone	0.9990	7.1	11.2	95 ± 7	0.34	1.0
β-Estradiol	0.9989	8.5	9.8	86 ± 6	0.52	1.6
Ethinylestradiol	0.9991	6.6	10.7	110 ± 9	0.49	1.5
α-Estradiol	0.9987	7.7	12.0	87 ± 6	1.09	3.3

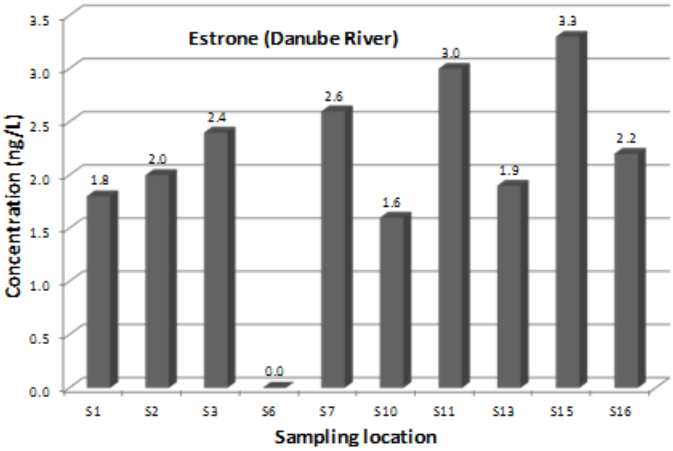

Fig. 3. Estrone concentration (ng/L) detected along the Danube River

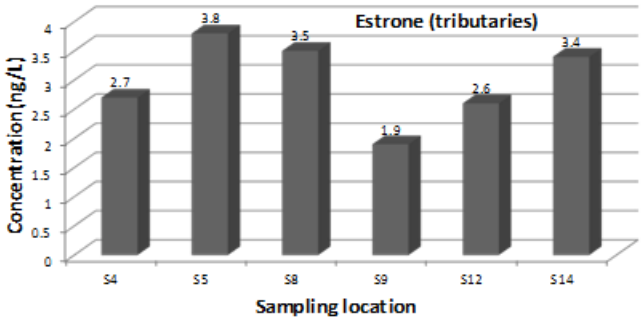

Fig. 4. Estrone concentration (ng/L) detected in Jiu, Olt and Arges tributaries

Instrumental LOQs were determined by injecting decreasing concentrations of hormone solutions until a S/N of 10 was obtained (table 4). Intra-day and inter-day method precision was tested on 6 replicates by spiking 25 ng/L hormone mixture in surface water. Accuracy was tested also at 25 ng/L and the obtained analyte recovery analyte was situated between 78 and 110% with internal standard correction (table 4). Specificity was tested using ultrapure water which was subjected to SPE extraction and LC-MS analysis. No significant interferences were observed for any of the target analytes.

Estrogen hormones occurrence in Danube River and tributaries

Hormone levels in Danube River and three of its main tributaries was tested during a sampling campaign in October 2015, using the newly developed LC-ESI(-)MS/MS method with automated SPE extraction. Estrone was the only estrogen hormone found at concentration levels higher than its LOQ (1.0 ng/L) both in Danube and tributaries (fig. 3-4). In some of the sampling locations β-estradiol and Ethinylestradiol were detected (> LOD), but were not quantifiable (< LOQ), so these values are not reported. It is worthwhile to note that literature data indicate that β-estradiol can easily transform into Estrone in the environment by oxidation. This might be an explanation of the low levels of β-estradiol in the tested surface water [11]. All other tested estrogen hormones, namely Equilin, Estriole and β-Estradiol were not detected at all in any of the 16 analyzed samples. Low to moderate levels of Estrone (<LOQ ÷ 3.8 ng/L) were determined in both Danube and tributaries with higher values for the latter (fig. 3-4). The lower levels detected in the Danube are probably caused by the higher water volume when compared with that of the tributaries. Analyzing Jiu and Arges levels of Estrone it can be observed that downstream major cities of Craiova and Bucharest (S5, S14) concentration is higher when compared to upstream values (S4, S12) (fig. 4). This is easily explained by the contribution of these cities to surface water pollution. Estrone levels in Danube and tributaries are generally of the same magnitude order with those reported by other studies in Europe with levels from <0.3 to 4.9 ng/L [12, 13].

Conclusions

A sensitive, selective and accurate LC-ESI(-)MS/MS method coupled with automated SPE extraction was developed to detect six estrogen hormones from surface water of Danube and three main tributaries (Jiu, Olt and Arges). Surface water samples were concentrated 500 times using SPE on Oasis HLB cartridges which generated overall method LOQs between 0.2 and 3.3 ng/L. Method accuracy was good with recovery values between 78 and 110%. Estrone was the only hormone found above LOQ in Danube and tributaries with concentration levels from below LOQ (Danube) up to 3.8 ng/L (Jiu, downstream Craiova). Also detected, but below LOQ values were β-Estradiol and Ethinylestradiol. Estrone levels in Danube and tributaries are generally of the same magnitude order with those reported by other studies performed in Europe.

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