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RESEARCH ARTICLE

Determination of selected steroid compounds in sediment samples from Golden Horn Estuary (the Sea of Marmara, Turkey) using LC-ESI/MS-MS

Duygu Aydoğan[1*], Türkan Yurdun[2]

ORCID IDs: D.A. 0000-0003-2663-3001; T.Y. 0000-0002-2554-1204

[1] Department of Environmental Sciences, Institute of Pure and Applied Sciences, Marmara University, Istanbul, TURKEY
[2] Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Marmara University, Istanbul, TURKEY

***Corresponding author:** aydogannduygu@gmail.com

Abstract

One of the most important areas where environmental pollution creates concern is aquatic environments. Steroids have the potential to disrupt the physiological function of hormones by interfering with the endocrine system. In our study, analysis of 31 selected human/animal, plant, natural and synthetic hormone-steroids was performed with Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI/MS-MS) device after the extraction of sediment samples from 15 stations in Istanbul Golden Horn with acidic methanol in an ultrasonic bath. Among the physiologically active estrogens used in birth control pills, equilin (54.46-2201.00 ng/g), estriol (2265.13 ng/g only in H1 sediment sample), mestranol (82.34-335.82 ng/g); from progestogens progesterone (1.59-6.03 ng/g), pregnenolone (44.19-1704.54 ng/g), levonorgestrel (1.55-7.78 ng/g); androgens 4-androstenedione (19.91-22.71 ng/g), androsterone (72.66-467.56 ng/g), testosterone (12.54-16.19 ng/g); cholestanone (157.57-1163.07 ng/g), coprostanol+epicoprostanol (42.82-103.26 ng/g) from human and animal wastes, and campesterol (143.86-1423.94 ng/g) from phytosterols were detected. As a result of the analysis, steroids were found in all sediment samples. Coprostanol+epicoprostanol and cholestanone, which are biomarkers of fecal contamination, are present in all of the sediment samples. Our study is the first to evaluate the presence of possible steroid hormones that pose a risk to organisms and ecosystem health in Golden Horn sediments.

Keywords: Estuarine system, hormones, sediment, LC-ESI/MS-MS, steroid, endocrine disrupting

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Introduction

It is almost impossible to remove organic compounds found in many products used in agricultural, domestic and industrial areas with traditional wastewater treatment methods. For this reason, the concern about the existence and future of organic micropollutants in aquatic ecosystems is increasing day by day (Mulabagal *et al.* 2017). Organic compounds reach aquatic ecosystems as a result of agriculture and livestock activities (Jauković *et al.* 2017), urban and industrial discharges (Venturini *et al.* 2015) (pharmaceuticals, pesticides, metals, steroid hormones, and etc.), atmospheric degradation (Zhang *et al.* 2014). One of these dangerous micropollutants, sterol and steroid hormones, have acute and chronic toxic effects on both humans and animals (Jauković *et al.* 2017). It has been proven by research that such compounds disperse into the environment and have effects wider than originally anticipated. As a result of the presence of steroid chemicals in the environment, there have been concerns about abnormal physiological processes, reproductive disorders, increase in cancer rate, development of antibiotic-resistant bacteria, and increased toxicity of chemical mixtures (Sojinu *et al.* 2012). For example; β-sitosterol, one of the plant sterols, causes reproductive dysfunctions in fish; deteriorating effects of phytosterols on the endocrine system and metabolism in European skunks have been detected (Jauković *et al.* 2017).

It is known that sewage treatment plant wastewater contains pollutants that are estrogenic for aquatic animals. Sex steroids remain in the ecosystem for a long time, bioaccumulating in some animals and becoming biomagnified along the food chain. Feminization was observed in male fish exposed to these estrogenic compounds, and secondary male sex developments were observed in female fish exposed to androgen and progestagen (Wangmo *et al.* 2018). Since it has negative effects on the functioning of feminization and endocrine metabolism in aquatic organisms, its long-term effects are expected since it changes the reproduction and development processes of animals (Praveena *et al.* 2016). These natural steroids secreted from the adrenal cortex, testis, ovary and placenta, are involved in the formation of secondary female character, secondary male character, emotional turmoil, surgery, hunger, etc. They play a role in responding to stressors, reproduction, salt-water and electrolyte balance (Ying *et al.* 2002). It is possible for steroids to undergo biotransformation through enzymes in the organism and be eliminated from the body through urine and faeces, and their transition to the natural environment through industrial and domestic wastewater as a result of drug production processes and drug use (Gomes *et al.* 2004). Wastes containing estrogen, androgen, progesterone as well as synthetic hormones is used as a fertilizer source in agricultural areas, thus hormone transports from agricultural areas as a result of irrigation and precipitation events have been documented (Sangster *et al.* 2015).

The sediment content acts as a kind of identity of the aquatic environment. While providing a living environment for living things, it also provides a carrier environment in terms of pollutants (Chiang *et al.* 2020). Because of their preference for binding to solid matrices, steroids can accumulate in surface water sediments (Gomes *et al.* 2004), and there is a certain level of saturation for pollutants that accumulate within the sediment layer. The sediment layer, which reaches this saturation level, releases these pollutants back into the water after a while (Lijklema *et al.* 1993).

Since sediments have the feature of storing this pollution; their content becomes important. So far, no study on the investigation of steroid hormones with sediment samples taken from Istanbul Golden Horn has been found in the sources. In this study, detailed data on the pollution caused by steroid hormones were revealed by LC-MS/MS analysis of sediment samples taken from 15 different stations from Istanbul Golden Horn. In this way, an insight was provided on the effects of biological pollution caused by micro-pollutants in the short and long term, not only individually, but also synergistically, additively and antagonistically.

Materials and Methods

Chemicals and reagents

Depending on the frequency of use and detection in environmental samples, hormones to be analyzed in sediments were determined. In this study, a total of 31 steroid hormones and sterols were selected. Hormones: 17-α –ethinylestradiol (Dr. Ehrenstorfer GmbH), estriol (Dr. Ehrenstorfer GmbH), estrone (Dr. Ehrenstorfer GmbH), levonorgestrel (Dr. Ehrenstorfer GmbH), mestranol (Cayman Chemical Company), norethindrone, equilin (Dr. Ehrenstorfer GmbH), 11-deoxycorticosterone, 11-deoxycortisol, 17-α-OH-progesterone, 4-androstenedione, 17-α-pregnenolone, aldosterone, androsterone, corticosterone, cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAs), dihydrotestosterone, 17β-e stradiol, pregnenolone, progesterone, testosterone; Human / animal sterols: cholesterol (Cayman Chemical Company), 5α-cholestan-3-one (cholestanone) (Alfa Aesar), 5β-cholestan-3α-ol (epicoprostanol) (Sigma-Aldrich), 5β-cholestan-3β-ol (coprostanol) (Sigma-Aldrich), 5α-cholestan-3β-ol (cholestanol) (Alfa Aesar), desmosterol (Cayman Chemical Company); Plant sterols: campesterol (Cayman Chemical Company), stigmasterol (Supelco). Standard materials, whose company names are not given, were included in kit (JSM-CL-6500, Sem Lab. Cih. Paz. San. and Tic. Inc., Turkey).

HPLC grade methanol from Riedel-de-Haen and formic acid from Lachema cat.nr. 30587 (Czech Republic) were obtained. Individual standard solutions were prepared at 1 mg/mL. Methanol was used as dilution solvent to prepare working standards and they were diluted to 1 µg/mL. All samples were stored at -20°C.

Sampling Area

Istanbul Golden Horn (Figure 1) is 7.5 km long with a width varying between 150-900 meters. It is a horn-shaped water estuary, which extends in the northwest-southeast direction, has a surface area of 2.5 km^2 and a maximum depth of 42 m. at its mouth (Dursun *et al.* 2018).

Sample Collection

Sediment samples were taken in July 2014. Sampling points were determined with a 12-channel ETRAX-Garmin GPS. Golden Horn Estuary (H) sediment samples were taken using the standard 0.1 m^3 capacity Van Veen sediment grab bucket. It was taken from 15 sampling points in Golden Horn Estuary (Figure 1). Wet samples were dried at 45°C and stored at -20°C until analysis.

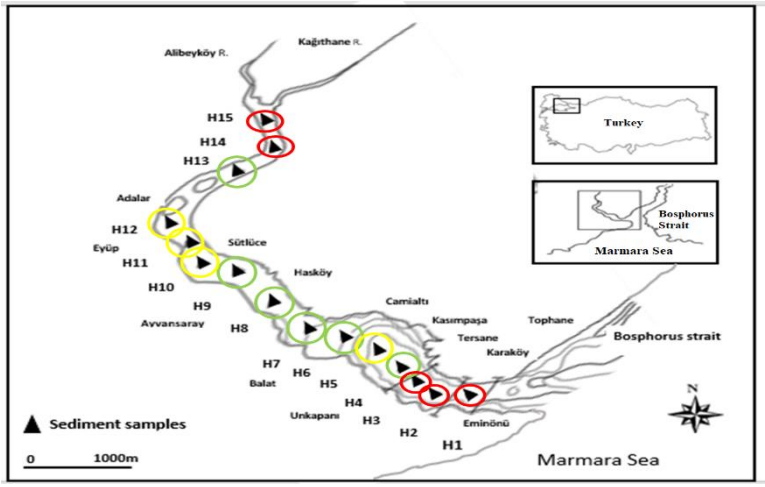

Figure 1. The locations of the sampling stations in Golden Horn Estuary
Green circles show total steroid pollution load between 0-3000 ng/g dw sediment, yellow circles show total pollution load between 3000- 3600 ng/g dw sediment, red circles show total pollution load higher than 3600 ng/g dw sediment.

Sample Extraction

One gram of dried sediment was weighed, added by 5 mL of methanol with 0,1% formic acid, vortexed (LMS VTX-3000L 20W Harmony Mixer Uzusio) for 1 min and sonicated (Elma Ultrasonic LC30) for 10 min. It was then centrifuged (Hettich Zentrifugen D-78532 Tuttlingen) at 4000 rpm for 10 min. Supernatant was transferred to the glass tube. This process was repeated two more times and repeated three times in total. The clear solution collected in the glass tube was evaporated to dryness in a 40°C heater (Stuart SBH130D) under gentle nitrogen flow. Residues were dissolved using 50 µL acetone and vortexed for 1 min. Then 950 µL of methanol was added and vortexed for 1 min again. It was centrifuged for 10 min at 4000 rpm. The clear part (supernatant) is transferred to the vial for injection to LC-ESI/MS-MS.

LC-ESI/MS-MS Analysis

Analyses of sediments were performed on Agilent Infinity 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of binary pump (G4220A), column compartment (G1316C) and autosampler (G7167B) coupled with 6470 triple quadrupole mass spectrometry (6470A, Agilent Technologies, Santa Clara, CA, USA). For the measurement of the concentrations of hormones in sediment specimens, CE-IVD certified validated Jasem Steroid Hormones LC-MS-MS analysis kit (JSM-CL-6500) and Jasem Steroid Hormones LC-MS-MS analysis method were used (Sem Laboratuar Cihazları Pazarlama San. and Tic. Inc., Istanbul, Turkey). Kit components applied throughout the analyses were as follows: analytical column specified for the analysis of steroid hormones, stable isotope labelled steroid hormones solution as internal standards (IS: estrone-2,3,4-^{13}C3, progesterone-D9, cortisol-D4, 11-deoxycortisol-D5, testosterone-^{13}C3, 17-hydroxyprogesterone-D8, DHEA-D5, androstendione-^{13}C3, dihydrotestosterone-D3, aldosterone-D7, DHEAS-D6). Positive and negative electrospray ionization (+ESI/-ESI) in dynamic multiple-reaction monitoring (dMRM) mode was simultaneously implemented for the MS-MS detection of the hormones. All the targeted hormones were ionized and detected in positive polarity with exception of DHEAS applied in negative polarity. HPLC system was operated to inject 20 µL of treated calibrators/samples into the analytical column which was maintained at 50°C. Steroid hormones analysis were performed with gradient system of mobile phases. The total running time was 21.0 min. The mass spectrometer settings of the kit were as follows; drying gas temperature 350˚C, drying gas flow 11 L/min, nebulizer pressure 30 psi, sheath gas temperature 400˚C, sheath gas flow 11 L/min, capillary voltages were 5500 and 3000 V for positive and negative respectively with 500 V nozzle voltage for both of polarities. The MS-MS detections were accomplished by product ion transitions created with collision-induced dissociation (CID) of corresponding precursor ion. According to the analysis kit, MRM transitions of the hormones and assigned IS were monitored at optimum fragmentation voltages (FV) which represented common value for each precursor ion-product ion mass transitions and optimum collision energies (CE) which indicated specific value for each product ions in term of voltage (V) unit. Quantification of the analytes were conducted by means of calibration curves made on the concentrations of the calibrators with compensating for matrix effect and procedural losses according to yields of the assigned IS. Data acquisition and quantification were carried out using Agilent MassHunter Acquisition and Quantitative Analysis software programs, respectively (Table 1).

Table 1. Values of steroid compounds mass spectrometer parameters

Steroids	Time (min)	Mw (g/mol)	Precursor ion (m/z)	Precursor ion Product ion (m/z)	Fragmentor (V)	Collision Energy (V)
Aldosterone	3.22	360.44	361.2 $[M+H]^+$	361.2→343.1	110	12
				361.2→315.1	110	18
Cortisol	3.80	362.46	362.9	362.9→120.7	110	20
				362.9→96.8	110	24
Estriol	4.03	288.38	271.2 $[M-H2O+H]^+$	271.2→253	80	0
				271.2→132.7	80	15
DHEAS (Dehydroepiandrosterone sulfate)	4.80	368.5	366.7 $[M-H]^-$	366.7→96.7	120	30
Corticosterone	4.93	346.5	346.9	346.9→328.6	110	10
				346.9→120.6	110	20
11-Deoxycortisol	5.12	346.461	346.9	346.9→108.5	120	26
				346.9→96.6	120	20
Estradiol	5.73	272.4	255.1 $[M-H_2O+H]^+$	255.1→158.9	90	12
				255.1→133.0	90	14
Testosterone	6.03	288.42	289.1 $[M+H]^+$	289.1→108.9	110	20
				289.1→96.9	110	18
Norethindrone	6.09	298.419	299.2 $[M+H]^+$	299.2→231.2	120	10
				299.2→109	120	24
Equilin	6.15	268.3	251.1 $[M- H_2O+ H]^+$	251.1→209	120	15
				251.1→195.3	120	15
11-Deoxycorticosterone	6.37	330.5	330.9	330.9→108.8	110	24
				330.9→96.8	110	20
17α-Ethynyl estradiol	6.37	296.4	279.3 $[M- H2O+ H]^+$	279.3→159	80	12
				279.3→132.9	80	8
Estrone	6.38	270.366	271.1 $[M+H]^+$	271.1→252.9	90	4
				271.1→132.8	90	20
4-Androstenedione	6.47	286.4	287.1 $[M+H]^+$	287.1→97.1	140	19

Table 1. Continued

DHEA (Dehydroepiandrosterone)	6.57	288.42	270.9 [M- H2O+ H]$^+$	270.9→252.9 270.9→96.8	90 90	6 20
17α-Hydroxyprogesterone	6.84	330.5	331.1 [M+H]$^+$	331.1→97.1	130	23
Dihydrotestosterone	7.21	290.44	290.9	290.9→272.9 290.9→254.8	130 130	8 10
Levonorgestrel	7.22	312.46	313.2 [M+H]$^+$	313.2→245.2 313.2→109	120 120	10 24
Androsterone	8.09	290.44	272.9[M- H2O+ H]$^+$	272.9→254.8 272.9→146.8	120 120	8 16
17α-Hydroxypregnenolone	8.64	332.5	315.2 [M- H2O+ H]$^+$	315.2→297.2 315.2→279.1 315.2→255.2	100 100 100	8 10 10
Progesterone	8.64	314.469	314.9	314.9→108.8 314.9→96.8	110 110	20 18
Pregnenolone	8.67	316.4776	298.9 [M- H2O+ H]$^+$	298.9→280.9 298.9→160.9	90 90	4 14
Mestranol	9.87	310.4	311.3 [M+H]$^+$	311.3→121 311.3→91	100 100	15 46
Desmosterol	14.45	384.64	367.3 [M- H2O+ H]$^+$	367.3→161.2 367.3→104.9 367.3→95	120 120 120	12 46 27
Epicoprostanol	15.67	388.66	371.5 [M- H2O+ H]$^+$	371.5→109 371.5→95 371.5→80.9	130 130 130	21 24 34
Coprostanol	15.67	388.67	371.5 [M- H2O+ H]$^+$	371.5→109 371.5→95	120 120	18 24
Campesterol	15.97	400.7	383.5 [M- H2O+ H]$^+$	383.5→161.1 383.5→147.1 383.5→95.1	110 110 110	15 18 30
Cholestanone	16.12	386.7	387.4 [M+H]$^+$	387.4→369.4 387.4→243.3	120 120	6 8

Results and Discussion

In this study, the analysis of steroids in Golden Horn sediment samples was carried out with the LC-ESI/MS-MS technique. MS parameters are shown in Table 1. The standard curves of steroid hormones and sterols were linear in concentration ranges 50, 100, 250 and 500 ng/mL. The method performance parameters for steroid hormones and sterols were shown in Table 2. Alsothe recovery studies were prepared by adding 100 and 500 ng/g of each standard solution to the sediment samples before extraction and they were left to dry at room temperature for one night. Then they were applied extraction procedure.

This is the first study to investigate the structure and residues of human/animal and plant sterols in the samples collected from Istanbul Golden Horn. Of the 31 monitored steroids, 13 were detected (Table 3). Cholesterol, cholestanol and stigmasterol could not be analyzed because they could not produce stable ions that improve quantification in electrospray ionization (ESI) technique. Mineralocorticoid derivative 11-deoxycorticosterone, aldosterone; glucocorticoid derivative 11-deoxycortisol, corticosterone, cortisol; estrogens 17-alpha-ethinylestradiol, 17β-estradiol and estrone; Androgens 17 α-OH-pregnenolone, dehydroepiandrosterone (DHEA), DHEAS, dihydrotestosterone; progestogens 17- α-OH-progesterone, norethindrone; desmosterol in sediment samples were below the limit of detection.

In the study, six estrogens, equilin, estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethynylestradiol (EE2) and mestranol were determined in sediment samples collected. Equilin is not normally found in females and is the only human estrogen hormone replacement therapy drug derived from the urine of pregnant mares. Equilin used together with E1 in formulations was detected in the Golden Horn sediments in the range of 54.46–2201.00 ng/g. H1 station has the lowest concentration value quite different from other stations. The highest concentration for the hormone equilin was measured at the H15 station as 2201.00 ng/g. EE2, a synthetic estrogen with strong biological effects, found in almost all oral contraceptive pills, could not be detected in sediment samples. Mestranol, another estrogen hormone used in menopausal hormone therapy and the treatment of menstrual disorders, was in the range of 82.34-335.82 ng/g, the highest mestranol concentration was detected at the H4 station. E1, E2 and E3, which are endocrine disrupting compounds, are among the natural estrogens and are excreted by humans and animals and thus released into rivers and seas. Unlike other estrogens, E3 hormone was detected as 2265.13 ng/g, which was quite high above the detection limit in only one station (H1), in the Golden Horn sediment samples. The E3 values in the sediments of the other 14 stations were below the detection limit value. Likewise, E1 and E2 were analyzed below the detection limit value in the sediment samples.

Table 2. Method performance parameters (n=3): recoveries at two concentration levels, method repeatability (relative standard deviations, RSDs), method linearity (correlation coefficients, r^2), limits of detection (LODs) and quantification (LOQs)

Steroid Hormone/Sterols	Recovery, % (RSD, %) Spiking level 100 ng/g	Recovery, % (RSD, %) Spiking level 500 ng/g	r^2	LOD (ng/g)	LOQ (ng/g)
Estrogens					
Estriol	80.8 (5.0)	82.9 (3.8)	0.9979	10.91	36.35
17-α Ethinylestradiol	86.7 (18.7)	112.4 (5.3)	0.9978	12.07	40.23
Estradiol	100.2 (12.2)	99.7 (13.6)	0.9995	7.09	23.63
Estrone	99.5 (9.8)	99.1 (12.2)	0.9991	12.23	40.77
Mestranol (Synthetic)	87.1 (7.5)	78.7 (6.9)	0.9991	8.11	27.02
Equilin (Synthetic)	79.1 (8.9)	94.8 (8.2)	0.9987	12.35	41.15
Androgens					
Androstenedione	82.8 (15.1)	66.8 (14.6)	0.9994	11.91	39.72
Androsterone	88.8 (5.4)	89.7 (4.5)	0.9987	6.35	21.16
Testosterone	101.0 (7.9)	93.1 (6.2)	0.9996	8.61	28.71
DHEA	87.3 (14.4)	64.7 (7.3)	0.9991	9.04	30.13
DHEAS	80.0 (6.1)	61.5 (6.4)	0.9984	4.35	14.51
Dihydrotestosterone	98.3 (12.3)	98.8 (3.5)	0.9993	4.67	15.55
Progestagens					
Pregnenolone	73.1 (4.0)	64.1 (7.0)	0.9998	11.05	36.85
Progesterone	87.7 (11.2)	82.5 (4.4)	0.9953	7.70	25.68
17-α-OH-pregnenolone	100.1 (5.4)	101.8 (5.1)	0.9915	7.79	25.96
17-α-OH-progesterone	93.2 (7.0)	100.5 (1.8)	0.9997	2.54	8.48
Levonorgestrel (Synthetic)	96.3 (12.6)	106.9 (17.3)	0.9999	12.78	42.60
Norethindrone (Synthetic)	89.2 (11.3)	71.4 (14.3)	0.9998	5.97	19.91
Fecal Sterols					
Coprostanol+ Epicoprostanol	96.4 (10.0)	60.1 (13.2)	0.9900 0.9995	9.41	31.37
Cholestanone	98.9 (5.8)	103.9 (5.5)	0.9986	11.22	37.40
Plant sterols					
Campesterol	108.5 (1.9)	71.8 (18.2)	0.9932	9.80	32.66
Desmosterol	94.9 (5.1)	98.4 (2.9)	0.9985	9.24	30.80
Glucocorticoids					
Deoxycortisol	80.7 (1.4)	74.4 (6.4)	0.9991	6.40	21.32
Cortisol	62.5 (3.6)	67.2 (8.4)	0.9991	4.80	15.99
Mineralocorticoids					
Corticosterone	70.6 (18.3)	64.5 (5.0)	0.9983	4.27	14.24
Deoxycorticosterone	99.7 (15.7)	100.0 (4.0)	0.9999	11.94	39.80
Aldosterone	77.7 (5.4)	58.3 (19.3)	0.9997	6.49	21.62

Table 3. Steroid concentrations in Golden Horn sediment samples (ng/g)

Steroids / Stations	4-Androste nedione	5-alpha-cholestan-3-on	Androsterone	Campesterol	Coprastanol+ Epi koprastanol	Equilin	Estriol	Levonorgestrel	Mestranol	Pregnenolone	Progesterone	Testosterone	Total Steroids
H1	20.13	827.66	83.74	877.75	94.51	54.46	2265.10	7.78	153.74	59.28	3.29	13.64	4461.11
H2	20.02	549.66	142.37	1423.90	77.88	1305.50	<LOQ	5.96	279.58	112.74	2.94	13.84	3934.43
H3	20.05	938.40	229.91	686.04	96.86	1101.80	<LOQ	5.09	335.36	195.68	5.65	14.52	3629.33
H4	20.23	291.59	189.07	438.53	65.03	1109.70	<LOQ	6.35	335.82	157.97	2.82	13.62	2630.70
H5	19.91	332.14	111.40	423.12	61.28	2064.60	<LOQ	8.12	236.45	129.3	3.11	13.76	3403.20
H6	20.41	161.46	104.17	<LOQ	42.82	1110.90	<LOQ	5.07	126.96	160.81	1.90	12.54	1747.02
H7	20.07	268.18	72.66	224.29	46.44	1140.40	<LOQ	5.08	155.88	169.12	1.59	12.82	2116.50
H8	20.44	255.50	112.10	143.90	45.62	960.70	<LOQ	3.45	205.70	418.00	2.71	14.33	2182.32
H9	20.41	157.57	276.63	417.18	45.29	1323.70	<LOQ	3.29	188.27	126.61	2.72	14.93	2576.56
H10	20.21	444.64	320.64	365.38	52.81	1794.80	<LOQ	1.55	176.89	97.10	3.07	14.89	3291.93
H11	20.64	638.44	362.77	415.19	73.89	1741.00	<LOQ	2.44	190.82	107.78	4.99	16.19	3574.13
H12	20.86	255.86	293.07	169.77	49.42	1833.70	<LOQ	3.25	243.51	117.22	3.05	13.62	3003.4
H13	21.20	376.81	168.03	275.76	43.21	1799.60	<LOQ	3.13	82.34	44.19	1.77	13.38	2829.45
H14	22.71	1163.1	467.56	702.44	103.26	1274.60	<LOQ	3.00	186.36	120.45	6.03	14.10	4063.59
H15	20.89	752.47	96.08	503.17	80.35	2201.00	<LOQ	2.64	172.77	1704.50	4.55	12.88	5551.34

According to the data obtained, the concentration of testosterone, an androgen steroid hormone, in the sediment samples was homogeneous at all stations, but these values varied between 12.54-16.19 ng/g. The average testosterone concentration value is 13.94 ng/g. The highest testosterone value was determined as 16.19 ng/g at H11 station. In addition, secondary and tertiary high concentration data measured 14.93 ng/g in H9 and 14.52 ng/g in H3.

Testosterone is metabolized in the liver to androsterone, which is not biologically active. Androsterone (3α-hydroxy-5α-androstan-17-one), a weak androgen hormone, was measured as 72.66-467.56 ng/g at Golden Horn sampling stations. The mean androsterone concentration was 202.01 ng/g. To our knowledge, no studies have detected androsterone in sediment samples. Our study is the first to detect androsterone in sediment samples. Weak androgen steroid hormone 4-androstenedione concentration was found to be in the range of 19.91-22.71 ng/g, showing close values at all stations. Androsterone was measured at maximum 6.80 ng/g in Lake Taihu sediment samples in China (Arima *et al.* 1969) reported that cholesterol, sitosterol and stigmasterol are degraded to progesterone and androstenedione by common soil bacteria. Owen *et al.* 1978 showed that the steroid synthesizing bacteria *E. coli* converts cholesterol from human feces to cholestenone and androstenedione. Environmental androgen reports mainly focused on paper mill wastewater. In the study investigating androgens, which are considered as environmental endocrine disruptors, androstenedione was found in the Fenholloway river sediment, where wastes of a paper mill were disposed, at concentrations of 0.7±0.2 µg/L, and progesterone, a biosynthetic precursor of androstenedione, as 48.8±7.0 µg/L, much higher than the amounts in the water column. The presence of androgens and androgen precursors in river water and sediment is thought to contribute to the masculine phenotype of the female mosquitofish (Gambusia holbrooki) in the Fenholloway River (Jenkins *et al.* 2003).

Pregnenolone values varied between 44.19-1704.54 ng/g at the Golden Horn sampling stations and were found to be quite high (1704.54 ng/g) in the H15 station compared to other stations.

Progesterone concentrations from progestogen class steroids were measured between 1.59-6.03 ng/g, with an average concentration value of 3.35 ng/g. The highest progesterone concentration was determined as 6.03 ng/g at H14 station. Other high concentration values were determined as 5.65 ng/g at station H3 and 4.99 ng/g at station H11. Levonorgestrel, another synthetic progesterone, was determined as 1.55-8.12 ng/g, the highest levonorgestrel concentration was measured at the H5 station as 8.12 ng/g. The mean levonorgestrel concentration is 4.41 ng/g.

Cholestanone, a potentially toxic compound, was detected in the Golden Horn sediment samples at very different concentrations in the range of 157.57-1163.07

ng/g. The station where 5-alpha-cholestan-3-one, which was determined as an average of 494.23 ng/g, was measured at the highest value, was station H14.

Coprostanols originate from sewage discharges in the area. Coprostanol-Epicoprostanol values, one of the sterols found in human and animal feces, ranged from 42.82-103.26 ng/g in the Golden Horn sediments. The highest coprostanol-epicoprostanol value was measured at H14 station as 103.26 ng/g, it was determined at lower values compared to other sterol hormones.

Campesterol (24-methylcholesta-5-en-3β-ol), another type of sterol, was measured in the range of 143.86-1423.94 ng/g, but this value was below the detection limit only at the H6 station.

In general, the general distribution of hormones in the Golden Horn sediment samples is as follows: Equilin > cholestanone > campesterol > pregnenolone > mestranol > andosterone > estriol > coprastanol-epicoprastanol > 4-androstenedione > testosterone > levonorgestrel > progesterone. The highest concentration of steroids was H15 (5551.34 ng/g), and the lowest concentration was H6 (1747.02 ng/g). There was not a single station where no steroid was measured.

When conducting research on the future of aquatic environments, information such as examining sediment layers, determining their content and identity of the environment is required. Steroid hormones are involved in many studies in which the river and sea sediments in the world are investigated in terms of organic pollutants, due to their negative effects on ecological life, their toxicity, their interference with the endocrine systems of living things, their tendency to feminization and masculinization, and their extinction. In this study, the presence, distribution and quantification of 31 steroids were investigated by sampling from 15 different stations to examine Istanbul Golden Horn sediments. On the other hand, natural estrogen, E3 was found to be quite high as 2265.13 ng/g only in the H-1 sediment sample. The highest concentrations of E1, E2, EE2 were measured as 1.069 μg/L, 5.25 μg/L, 1.65 μg/L, respectively in Golden Horn by Korkmaz *et al.* (2020) in the bottom and surface water samples. In winter, the concentrations of hormones were found to be higher in bottom waters than in surface waters (Korkmaz *et al.* 2020). In the surface sediments of Latin America (SSES, Santos and São Vicente estuarine system/Brazil), the amount of E3 is between 20.9 ng/g and 694.2 ng/g, E2 and EE2, almost all sampled detected at the points (Pusceddu *et al.* 2019). The highest E2 concentration was 23.9 ng/g, while the highest EE2 levels were 86.3 ng/g. It has been reported that this situation is partially related to domestic sewage discharge. In the study conducted in Langat River/ Malaysia, E3 was measured as 2.4e-5±0.02 μg/g (Praveena *et al*. 2016). E3 is one of the hormones included in the Safe Drinking Water Act Contaminant Candidate List 4 (CCL4), which the EPA includes 8 estrogens under the Safe Drinking Water Act EPA 2016). The presence of E3 indicates human and animal feces (Praveena

et al. 2016). E3 in another study conducted by Streck (2009), it was measured as 3.37 ng/g in sediment samples. In the sediments of the Songhua River in northeastern China, E3 was detected at a low level of 1.09 ng/g in one sample out of 25 samples. In the same study, E1 was found to be 0.84-17.8 ng/L in all water samples, while it was found to be 0.50-3.05 ng/g in sediment samples at lower levels. E2 was found below the detection limit value in water samples and 0.11-1.16 ng/g in 3 sediment samples (Zhang *et al.* 2014).

Secondarily, 877.75 ng/g campesterol was detected in the same H1 station at Golden Horn. Campesterol value was measured as 9.0-151 ng/g in Barigui River sediments and the source of this sterol was stated as herbivorous animals (Machado *et al.* 2014). Therefore, the presence of campesterol can be attributed to wastewater from livestock activities.

As a type of estrogen hormone, equilin was detected quite low in H1 station, unlike other sampling stations. While 54.46 ng/g value was the lowest concentration, this value was 30-35 times higher in other stations. The highest concentration of equilin was determined as 2201 ng/g in the lowest station H15. In studies conducted in Potomac River sediments and fish tissues in Virginia, USA, equiline was found at a rate of 18% (Arya *et al.* 2017). In addition, mestranol, the other type of estrogen, was found at very close concentrations at H3 and H4 stations, but at H4 it was measured as 335.82 ng/g. The low solubility of mestranol in water allows it to be absorbed into the sediment. It was detected for the first time in Danube II river sediments as 10±3 ng/g in only one station (Matić *et al.* 2014). In the other study of the same authors, mestranol was found in only two of the samples (S1 sample 11±1 and S10 sample 19±3) (Matić Bujagić *et al.* 2016).

Cholestanone was measured at concentrations of 157.57-1163.07 ng/g in our study. This high value at H14 station was determined as 1163.07 ng/g. In another study, cholestanone was measured as 4.86 ng/g in the Rio De Plata estuary (Atlantic) sediments (Venturini *et al.* 2015). 5.16 µg/g Cholestanone was measured in the Guajará estuary in Brazil (Gomes *et al.* 2015). This value is 264 ng/g for Kuwait Doha and Sulaibihat gulf sediments (Lyons *et al.* 2015).

Testosterone, one of the androgen hormones detected in the study, was found to be the highest at 16.19 ng/g at the H11 station. The most observed androgens in aquatic environments are; originates from paper mill and farm animal feed wastewater, the most typical of which is testosterone (Aufartová *et al.* 2011). Testosterone concentration was measured as 9.67 pg/g in Alabama Perdido Estuary sediments (Mulabagal *et al.* 2017).

Levonorgestrel is combined with estrogen to prevent pregnancy. Levonorgestrel and progesterone as progestogen hormones were observed at stations H5 and H14. The highest levonorgestrel concentration was determined as 8.12 ng/g, while this

value was 6.03 ng/g for progesterone. Considering other studies, progesterone and levonorgestrel were measured as 22.3 pg/g and 3.44 pg/g, respectively, in Perdido sediments in Mexico (Mulabagal *et al.* 2017). In another study, 2.18 ng/g levonorgestrel and 6.82 ng/g progesterone hormone were detected in the sediment analysis (Streck 2009). In another sediment study conducted in Hunting Creek (Potomac River), striped killer fish exposed to progesterone and estrone had higher levels of steroid hormones than sediment, which were reported to develop a life cycle dependent on sexual maturation compared to white bass that were not exposed to these hormones (Arya *et al.* 2017). In the study of Jenkins *et al.* (2003) progesterone, a biosynthetic precursor of androstenedione, was found at a very high concentration of 48.8±7.0 µg/L. It has been reported that the paper mill as a source of high progesterone is an intermediate product as a result of microbial degradation of pine phytosterols in pulp waste released into the river (Jenkins *et al.* 2003).

Cholesterol is an important biological molecule and is the precursor of steroid hormones, vitamin D and bile acids. Cholesterol synthesized in hepatocytes is secreted into the gallbladder and then into the small intestine. It combines with dietary cholesterol in the human gut and is considered an excellent indicator of fecal contamination in sewage effluent with coprostanol, which is formed as a result of microbial conversion (Bull *et al.* 2002; Kenny *et al.* 2020). Studies on sediments are generally about metal analysis in Turkey (Okay *et al.* 2008; Çoban *et al.* 2009; Küçük and Topçu 2017; Ünlü and Alpar 2017; Kalaycı *et al.* 2021; Özşeker 2021; Kutlu *et al.* 2021). Studies on steroids in marine sediments are limited. In the evaluation of sewage pollution of sediments, the most reliable sterol ratios were coprostanol/(coprostanol+cholestanol);coprostanol/cholesterol; epicoprostanol/coprostanol has been reported (Matić Bujagić *et al.* 2016), the ratio of coprostanol to epicoprostanol, the isomer, is used to distinguish fecal contamination from humans and marine mammals. Human feces contains traces of epicoprostanol, which can also occur during anaerobic sewage treatment (Leeming *et al.* 1997).

In the study conducted in the Brazilian Barigui River sediments, sterols were examined by GC/MS method, coprastanol concentrations were measured as 13.6-447 ng/g, and epicoprostanol as 2.6-203 ng/g (Machado *et al.* 2014). In the Golden Horn estuary sediments, the highest coprostanol-epicoprostanol concentration was observed at H14 station as 103.26 ng/g. Coprostanol can be converted to epicoprostanol under anoxic conditions and cannot be found naturally in water sediments as an indicator of fecal pollution, an indicator of treated wastewater discharge (Frena *et al.* 2016).

In addition, some authors stated that in determining the pollution levels, levels between 10-100 ng/g dw of coprostanol indicate uncontaminated environments, and values greater than 100 ng/g dw indicate sewage pollution. They stated that 500 ng/g dw expresses intense sewage pollution (González-Oreja and Saiz-

Salinas 1998; Lyons *et al.* 2015). In this study, coprostanol and epicoprostanol could not have been chromatographically separated and were quantified as a sum. Coprostanol+epicoprostanol levels were 42.82-103.26 ng/g (mean: 65.24 ng/g). Of the 15 sites sampled one exceeded the 100 ng/g dw coprostanol threshold, indicating low sewage pollution at the H14 location (103.26 ng/g). Coprostanol levels measured in other authors' studies were quite high: Kuwait coastline sediments 29.2-2920 ng/g (Lyons *et al.* 2015). Patos Lagoon/Brazil surface sediments de 1,423 ng/g dw (Martins *et al.* 2007), Sochi, Black Sea Russia, Black Sea Ukraine and Bosphorus entrance to the Black Sea/Turkey sediments 54–5400 ng/g; 1-2600 ng/g, 12-440 ng/g respectively (Readman *et al.* 2005), Danube Basin/ Serbia 201-1939 ng/g1 (Matić Bujagić *et al.* 2016), surface sediments from Cienfuegos Bay, Cuba 10-5400 ng/g (Tolosa *et al.* 2014). In addition, since coprostanol is more stable because it is adsorbed to sediment, the fact that it is 0.02-0.22 μg/L in water samples, while it is quite high in surface sediments at 0.22-33 μg/g concentrations indicates that the Narragansett Bay/ USA is affected by sewage (LeBlanc *et al.* 1992).

Total steroid concentrations in the Golden Horn stations were determined in the range of 1747.02-5551.34 ng/g (Figure 1). The highest values were found at sediment sampling points H1 (4461.11 ng/g), H2 (3934.43 ng/g), H3 (3629.33 ng/g), H14 (4063.59 ng/g) and H15 (5551.34 ng/g) (Figure 2). H1, H2 and H3 stations are located in Sirkeci-Karaköy workplaces which densely populated areas and the daily working population is quite high. This situation may cause an excess of sediment steroid load. There was E3 in the sediments of H1 stations, also in the sediments of H2 and H3 stations where there was an excess of estrogenic equilin. The sediments of stations H14 and H15 also contain hight amounts of steroids. With the steroid load coming from Alibeyköy and Kağıthane streams, the possibility or absence of water circulation in the interior regions of Golden Horn can increase steroid loads.

Future studies on the Golden Horn/Istanbul sediments and pollution levels must be monitored by analysis. The presence of mestranol, equilin and estriol (urine estrogen) indicates pollution that origins from human. In addition, such compounds could potentially affect the marine food chain. This situation can cause feminization and intersex behaviors in fish (Jackson *et al.* 2019). Total steroids were found in the sediments of Guanabara Bay and coastal lagoons of the State of Rio de Janeiro in the range of 7.41-251 μg/g. Fecal pollution indicator coprostanol amount was determined as 36.2 μg/g (Santos *et al.* 2008). Likewise, in the summer surface samples of the Bilbao/Spain Estuary, coprostanol is the major sterol and was found at high levels as 18-293 μg/g (González-Oreja and Saiz-Salinas 1998). Grimalt *et al.* (1990) found cholestanol 0.25-16 μg/g, coprostanol 0.41-390 μg/g, epicoprostanol 0.04-5 μg/g, cholestanon 0.06-20 μg/g and coprostanone 0.7-120 μg/g on different sediment samples of Barcelona, Guadalquivir Delta, Santa Pola/Spain; Havana/Cuba. They reported that coprostanone levels would be a useful parameter in monitoring urban sewage

pollution, since the concentrations of coprostanol and coprostanone are higher than those of the 5α-epimers (Grimalt *et al.* 1990).

Our study is the first to reveal the steroid levels in the sediments obtained from 15 different points detected in the Golden Horn region of Istanbul. Our results were found to be less than 100 ng/g in the amount of coprostanol + epicoprostanol in the sediments, which are indicators of fecal contamination, according to the thresholds determined (only one sample out of 15 sediment samples is greater than 100 ng/g, 103.26 ng/g). Coprostanol concentrations above 500 ng/g were not found in the analyzed sediment samples (Table 3).

Considering the direct and indirect effects of steroids on the environment, animals and therefore on humans, the importance of organic pollution has been emphasized once again. It has gained great importance to examine wastewater treatment plants with water outlets, sediment samples and new studies to be made on organisms thought to be at risk, and to investigate and deepen the possible effects. It is thought that our results can also form the basis for new treatment processes to be planned for the future.

In the study of Martins *et al.* (2007), the abundance ratio of coprostanol in total sterol is between 50-80% is accepted as an indicator of pollution. For this reason, the pollution degree of Golden Horn was evaluated by using these ratios. Our current results in our study were found to be low in the range of 2.79-25.22 %. Although no pollution was observed in the Golden Horn sediment samples, it is recommended that the Golden Horn sediments be observed for pollution in the future (Table 4). It was possible to evaluate the sewer entrance and pollution points in the investigated area.

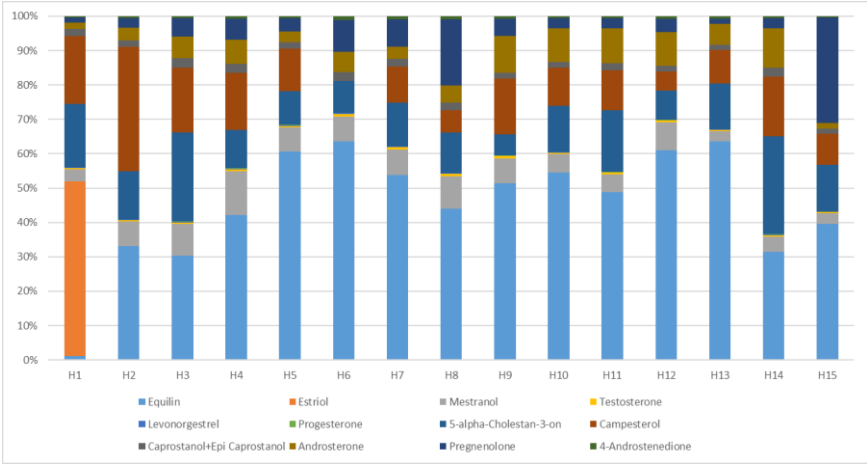

Figure 2. Distribution of steroid hormones in sediments of Golden Horn Estuary stations

Table 4. Concentration of individual sterols (ng/g dw) and selected ratio in surface sediments from Golden Horn Estuary, Istanbul

Sterols	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15
Cop.- Epi cop.	94.51	77.58	96.86	65.03	61.28	42.82	46.44	45.62	45.29	52.81	73.89	49.42	43.21	103.26	80.35
Mestranol	153.74	279.59	335.36	335.82	236.45	126.96	155.88	205.72	188.27	176.89	190.82	243.51	82.34	186.36	172.77
Campesterol	877.75	1423.94	686.06	438.53	423.12	<LOQ	224.29	143.86	417.18	365.38	415.19	169.77	275.76	702.44	503.17
Estriol	2265.13	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Cholestanone	827.66	549.66	938.40	291.59	332.14	161.46	268.18	255.47	157.57	444.64	638.44	255.86	376.81	1163.07	752.47
Σ-OLs	3391.13	1781.4	1118.26	839.38	720.85	169.78	426.61	395.20	650.74	595.08	679.9	462.70	401.31	992.06	756.29
% (cop+e-cop)/ Σ-OLs	**2.787**	**4.372**	**8.662**	**7.747**	**8.501**	**25.221**	**10.886**	**11.544**	**6.960**	**8.874**	**10.868**	**10.681**	**10.767**	**10.409**	**10.624**

% (cop+e-cop)/Σ-OLs: percentage of coprostanol + epicoprostanol in the sum of sterols; Σ-OLs: sum of sterols

As a conclusion, this is the first study to evaluate the presence of possible steroids and hormones that pose a risk to organisms and ecosystem health in the Golden Horn/Istanbul surface sediments. Coprostanol+epicoprostanol and cholestanon, which are biomarkers of fecal pollution, are present in all sediment samples. This indicates that human-induced, that is, faecal pollution, is present in the sediments, but not at high levels. The study constitutes the first data as an indicator of anthropogenic contamination in the studied area.

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Haliç (Marmara Denizi, Türkiye) sediment örneklerinde seçilen steroid bileşiklerinin LC-ESI/MS-MS yöntemiyle araştırılması

Öz

Çevre kirliliğinin endişe yarattığı en önemli alanlardan biri sucul ortamlardır. Steroidler, endokrin sisteme müdahale ederek hormonların fizyolojik işlevini bozma potansiyeline sahiptir. Çalışmamızda, İstanbul Haliç'te 15 istasyondan sediment örneklerinin ultrasonik banyoda asidik metanol ile ekstraksiyonundan sonra, Sıvı Kromatografi Elektrosprey İyonizasyon Tandem Kütle Spektrometresi (LC-ESI/MS-MS) cihazı ile seçilen 31 insan/hayvan, bitki, doğal ve sentetik hormon-steroidlerin analizi yapılmıştır. Doğum kontrol haplarında kullanılan fizyolojik aktif östrojenlerden equilin (54.46-2201.00 ng/g), estriol (sadece H-1 sediment örneğinde 2265.13 ng/g), mestranol (82.34-335.82 ng/g); progestojenlerden progesteron (1.59-6.03 ng/g), pregnenolon (44.19-1704.54 ng/g), levonorgestrel (1.55-7.78 ng/g); androjenler 4-androstenedion (19.91-22.71 ng/g), androsteron (72.66-467.56 ng/g), testosteron (12.54-16.19 ng/g); insan ve hayvan atıklarından kaynaklanan kolestanon (157.57-1163.07 ng/g), koprostanol+epikoprostanol (42.82-103.26 ng/g) ve fitosterollerden kampesterol (143.86-1423.94 ng/g) tespit edilmiştir. Analiz sonucunda tüm sediment örneklerinde steroidler tespit edildi. Fekal kontaminasyonun biyobelirteçleri olan koprostanol+epikoprostanol ve kolestanon tüm sediment örneklerinde mevcuttur. Çalışmamız Haliç sedimentlerinde organizma ve ekosistem sağlığı için risk oluşturan olası steroid hormonlarının varlığını değerlendiren ilk çalışmadır.

Anahtar kelimeler: Nehir ağzı sistemi, hormonlar, sediment, LC-ESI/MS-MS, steroid, endokrin bozucu

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