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Development and validation of an ultra-high performance liquid chromatographic high resolution Q-Orbitrap mass spectrometric method for the simultaneous determination of steroidal endocrine disrupting compounds in aquatic matrices

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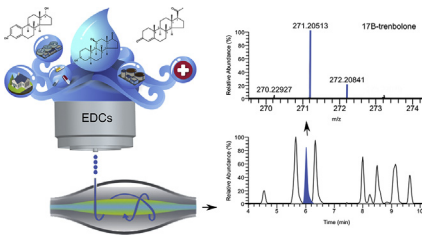
HIGHLIGHTS

- Optimised and validated UHPLC-HR-Q-Orbitrap-MS method.
- Simultaneous quantitative measurement of 70 steroidal EDCs.
- Very low empirical method detection and quantification limits.
- Applicability of method: robust EDC profiling of the marine environment.
- High identification potential because of various diagnostic ions.

GRAPHICAL ABSTRACT



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ABSTRACT

The lack of adequate strategies for monitoring endocrine disrupting compounds (EDCs) in the aquatic environment is emphasized in the European Water Framework Directive. In this context, a new UHPLC-HR-Q-Orbirtrap-MS multi-residue method was developed for the simultaneous measurement of 70 steroidal EDCs in two aquatic matrices, i.e. sea and fresh water. First, an instrumental APCI-UHPLC-HR-Q-Orbitrap-MS was devised for separating and detecting the EDC isomers and mass analogues, within 12.5 min per run. Next, an appropriate extraction was statistically optimised using a three-strep workflow (95% confidence interval, $p > 0.05$); including fractional factorial resolution IV, simplex lattice, and response surface methodological designs. The fitness-for-purpose of the method was demonstrated through successful validation at relevant environmental concentrations, i.e. the low nano- and picogram range. Method quantification limits ranged for the androgens ($n = 33$), oestrogens ($n = 14$), progestins ($n = 12$), and corticosteroids ($n = 11$) between, respectively, 0.13 and 5.00 ng L^{-1}, 0.25 and 5.00 ng L^{-1}, 0.13 and 2.50 ng L^{-1}, and 0.50 and 5.00 ng L^{-1}. Good linearity ($R^2 \geq 0.99$) and no lack of fit was observed (95% confidence interval, $p > 0.05$) for the 70 steroidal EDCs. In addition, good recovery (95–109%) and satisfactory repeatability (RSD < 8.5%, $n = 18$) and reproducibility (RSD < 10.5%, $n = 12$) were obtained. Finally, the applicability of the multi-residue method was demonstrated by measuring steroidal EDC in 28 sea water samples collected from four different locations during fall 2016 and winter 2017. Regarding

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the sea water samples, all the classes were ubiquitously present and included different metabolites, transformation product and or degradation products from the parent EDCs (n = 43).
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1. Introduction

The abundance of endocrine disrupting compounds (EDCs) in water is detrimental to the aquatic environment and its ecological health [1,2], resulting in severe consequences such as loss of animal habitats, reduction in biodiversity and intoxication - both acute and chronic - of organisms. For example, in case of fish, such as cyprinids and zebra fish, several negative effects have been reported. Not only do EDCs, such as the synthetic oestrogen 17α-ethinylestradiol or the androgen trenbolone, influence the fertility and reproduction capabilities of fish, they also impact the gender distribution [3–6]. In spite of the plethora of adverse effects that have been observed for many EDCs, only 17β-estradiol and 17β-ethinylestradiol have been included in the European watch list for water quality monitoring so far [7]. In order to further improve environmental quality standards, other EDCs, which have received little attention in the aquatic environment, need to be monitored as was recently recommended by Fent et al. (2015) [8].

EDCs mainly include steroidal and non-steroidal compounds. The non-steroidal EDCs comprise contaminants such as phthalates, phenols, antibiotics and polychlorinated biphenyls, whereas the steroidal compound group consists of androgens, oestrogens, progestins, and corticosteroids [9]. Due to the fact that steroidal compounds are the most potent endocrine disruptors in aquatic systems [10,11], these were the main focus within this work. The first steroidal subgroup concerns the androgens, which are the most abundant hormones found in effluents of wastewater treatment plants. These hormones originate from urinary excretion of humans and animals, whereby their presence is due to their usage for therapy and growth treatment [12]. The second steroidal subgroup, i.e. the oestrogens, and in particularly 17α-ethinylestradiol, are widely consumed as oral and non-oral contraceptives [13]. The third steroidal subgroup, i.e. the progestins, are extensively used for contraception and medical treatments and are consumed more than androgens and oestrogens. The last subgroup, i.e. the corticosteroids, are used to treat a variety of diseases, such as asthma, rheumatism, allergies and inflammation [14].

Up to now, studies mainly report on the occurrence of EDCs and related compounds in freshwater environments, whereas data for marine environments are relatively scarce [15,16]. The fresh water environments that have mainly been monitored for EDCs are riverine water, groundwater, drinking water, and wastewater [17–21]. These four major freshwater bodies suffer from contamination primarily due to local anthropogenic activities. As a result, only information on the occurrences of local EDCs is available [22]. As all water eventually ends up in the marine environment and in light of the above-mentioned effects, it is of utter importance to map the contamination status of marine waters as well. However, seawater analysis is complicated by the fact that EDCs prevail in the marine environment in the low ng L^{-1} range and that each EDC can occur in one or more of the following forms: parent EDCs, metabolites, transformation products, and or degradation products [8,23,24]. So far, only three EDCs, namely estrone, 17β-estradiol and 17α-estradiol, have been studied in the marine environment [16].

In this study, a method is presented that allows quantifying 70 target EDCs leaves also the possibility to screen for a virtually unlimited number of (un)known compounds in the marine environment. To realise this, an appropriate extraction and ultra-high performance liquid chromatographic high resolution Q-orbitrap™ mass spectrometric method (UHPLC-HR-Q-orbitrap™-MS) was developed for EDCs in marine waters. The UHPLC-part enables fast simultaneous separation of oestrogens, androgens, progestins and corticosteroids. Furthermore, the HRMS allows a reliable, selective and accurate target detection of the various EDC classes. The analytical method was validated according to CD 2002/657/EC [25], CD 2009/90/EC [26], Eurachem guidelines [27] and review articles [28,29] and eventually applied on real environmental samples.

2. Materials and methods

2.1. Chemicals and reagents

In this study, 70 steroidal EDCs were included (Table 1 and Table A.1.), which were purchased at Steraloids Inc (Newport, RI, USA) and Sigma Aldrich (St. Louis, MO, USA). The selected EDCs were based on relevant literature [15,17–20,30], and covered 4 classes, i.e. 33 androgens, 14 oestrogens, 12 progestins and 11 corticosteroids. The selected deuterated internal standards for each class were purchased at Steraloids (Newport, RI, USA) and Sigma Aldrich (St. Louis, MO, USA) and comprised 6 androgens, 5 oestrogens, 4 progestins, and 2 corticosteroids (Table 1).

Primary stock solutions and standard mixtures were prepared in methanol, thereby reaching concentrations between 0.01 and 1000 ng μL^{-1}. The solutions were stored in dark glass bottles at −20 °C. The organic solvents were of optima UPLC-MS grade, purchased from Fisher Scientific (Loughborough, UK). The inorganic salts, used to prepare reference seawater, were prepared according to ASTM D-1141 [31] and supplied by Sigma Aldrich (St. Louis, MO, USA). The C_{18} and H_2O-phillic divinylbenzene (DVB) Speedisks were purchased from Filterservice (Eupen, Belgium).

2.2. Instrumentation

The EDCs were chromatographically separated using an UHPLC system, consisting of an Ultimate 3000 XRS pumping system, coupled to an Ultimate 3000 RS column compartment and autosampler (Dionex, Amsterdam, The Netherlands). Chromatographic separation was achieved using reversed phase chromatography with gradient elution. Separation of EDCs was carried out using a Hypersil Gold column (1.9 μm, 100 × 2.1 mm) (Intersciences, Louvain-La-Neuve, Belgium) at a temperature of 45 °C. Furthermore, the mobile phase consisted of a mixture of water (Eluent A) and methanol (Eluent B) both containing 0.1% formic acid, pumped at a flow rate of 0.55 mL min^{-1}. The linear gradient program was as follows: 0 min, 40% B; 0–5.8 min, 40–65% B; 5.8–9.0min, 65–100% B; 9.0–10.5 min, 100% B; 10.5–10.6 min, 100-40% B; 10.5–12.5 min, 40% B. The injection volume was 2 μL.

The detection of EDCs was carried out using a Q-Exactive™ Benchtop HRMS (Thermo Fisher Scientific, San-Francisco, USA) fitted with an atmospheric-pressure chemical ionisation (APCI) source. Optimal positive and negative ionisation source working parameters were sheath gas flow 33 a.u. (arbitrary units), auxiliary gas flow 15 a.u., sweep gas flow 2 a.u., discharge current ± 4 kV, capillary temperature 250 °C, and vapour temperature 250 °C. The

Table 1
Instrumental performance of the target compounds included in the multi-EDC UHPLC-APCI-HR-Q-Orbitrap-MS method (mass accuracy was determined through 5 subsequent sample injections).

Compound	Elemental formula	IS	t_R (min)	Δ t_R (min)	Diagnostic ion for quantification	Other diagnostic ions for confirmation	Theoretical mass (*m/z*)	Empirical mass (*m/z*)	Mass Accuracy (Δppm)
Androgens									
Methandriol	$C_{20}H_{32}O_2$	e	6.34	0.01	$[M+H-2(H_2O)]^+$	$[M+H-(H_2O)]^+$	269.22638	269.22580	0.557
17α-trenbolone	$C_{18}H_{22}O_2$	b	5.52	0.02	$[M+H]^+$		271.16926	271.16867	0.406
17β-trenbolone	$C_{18}H_{22}O_2$	b	4.55	0.03	$[M+H]^+$		271.16926	271.16864	0.479
11β-hydroxyandrosterone	$C_{19}H_{30}O_3$	c	5.65	0.01	$[M+H-2(H_2O)]^+$	$[M+H]^+$, $[M+H-(H_2O)]^+$	271.20564	271.20498	0.479
Testosterone 17β-cypionate	$C_{27}H_{40}O_3$	d	9.82	0.01	$[M+H-C_8H_{14}O_2]^+$		271.20564	271.20506	0.369
Ethylestrenol	$C_{20}H_{32}O$	e	6.68	0.01	$[M+H-(H_2O)]^+$		271.24203	271.24549	0.811
17β-dihydroandrosterone	$C_{20}H_{34}O_2$	e	6.95	0.01	$[M+H-2(H_2O)]^+$		271.24203	271.24557	0.442
Androsterone	$C_{19}H_{30}O_2$	e	7.38	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$	273.22129	273.22058	0.659
Epi-androsterone	$C_{19}H_{30}O_2$	d	6.44	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$, $[M+H-2(H_2O)]^+$	273.22129	273.23823	0.915
19-nortestosterone	$C_{18}H_{26}O_2$	b	4.86	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	275.20056	275.20005	0.509
3β-androstanediol	$C_{19}H_{32}O_2$	f	8.53	0.01	$[M+H-(H_2O)]^+$		275.23694	275.22389	0.581
3α-androstanediol	$C_{19}H_{32}O_2$	f	8.94	0.01	$[M+H-(H_2O)]^+$		275.23694	275.20038	0.509
1,4-Androstadienedione	$C_{19}H_{24}O_2$	b	3.78	0.02	$[M+H]^+$	$[M+H-(H_2O)]^+$	285.18490	284.51761	0.527
11-ketoetiocholanolone	$C_{19}H_{28}O_3$	b	4.61	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$	287.20056	287.19993	0.522
Androstenedione	$C_{19}H_{26}O_2$	b	4.84	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	287.20056	287.19997	0.627
Mestanolone	$C_{20}H_{32}O_2$	e	7.29	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$	287.23694	287.23619	0.696
17α-testosterone	$C_{19}H_{28}O_2$	d	6.32	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	289.21621	289.21558	0.484
17β-testosterone	$C_{19}H_{28}O_2$	c	5.55	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	289.21621	289.21554	0.415
5α-dihydrotestosterone	$C_{19}H_{30}O_2$	e	6.65	0.01	$[M+H]^+$	$[M+H-n(H_2O)]^+$, n = 1,2	291.23186	291.23117	0.481
Norethindron	$C_{20}H_{26}O_2$	a	5.15	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	299.20056	299.19988	0.401
Methylboldenone	$C_{20}H_{28}O_2$	a	5.23	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	301.21621	301.21561	0.764
11-ketotestosterone	$C_{19}H_{26}O_3$	b	3.01	0.01	$[M+H]^+$		303.19547	303.19483	0.561
Formestano	$C_{19}H_{26}O_3$	b	2.10	0.01	$[M+H]^+$		303.19547	303.19473	0.759
Norethandrolone	$C_{20}H_{30}O_2$	e	7.02	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	303.23186	303.23125	0.495
Methyltestosterone	$C_{20}H_{30}O_2$	d	6.22	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	303.23186	303.23123	0.429
Trenbolone acetate	$C_{20}H_{24}O_3$	e	7.10	0.01	$[M+H]^+$		313.17982	313.17912	0.543
Ethynyl testosterone	$C_{21}H_{28}O_2$	d	6.24	0.01	$[M+H]^+$		313.21621	313.21551	0.415
Stanozolol	$C_{21}H_{32}N_2O$	e	6.77	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	329.25874	329.25798	0.881
Testosterone acetate	$C_{21}H_{30}O_3$	f	8.00	0.01	$[M+H]^+$	$[M+H-C_2H_3O_2]^+$	331.22677	331.22603	0.604
Fluoxymesterone	$C_{20}H_{29}FO_3$	b	4.55	0.01	$[M+H]^+$		337.21735	337.21656	0.623
Testosterone propionate	$C_{22}H_{32}O_3$	f	8.47	0.01	$[M+H]^+$		345.24242	345.24167	0.637
Chlorotestosteron acetate	$C_{21}H_{29}ClO_3$	f	8.25	0.01	$[M+H]^+$	$[M+H-Cl-C_2H_3O_2]^+$	365.18780	365.18699	0.548
Testosterone benzoate	$C_{26}H_{32}O_3$	f	9.05	0.01	$[M+H]^+$		393.24242	393.24153	0.610
Testosterone phenylpropionate	$C_{28}H_{36}O_3$	f	9.14	0.01	$[M+H]^+$	$[M+H-C_7H_8O]^+$	421.27372	421.27264	0.356
19-nortestosterone-17-decanoate	$C_{28}H_{44}O_3$	f	9.99	0.01	$[M+H]^+$	$[M+H-C_{10}H_{19}O_2]^+$	429.33632	429.33525	0.932
17β-trenbolone-d_3 (a)	$C_{18}H_{19}O_2d_3$		5.08	0.01	$[M+H]^+$		273.18181	273.18423	0.549
Nortestosterone-d_2 (b)	$C_{18}H_{24}O_2d_2$		4.86	0.01	$[M+H]^+$		277.21311	277.21256	0.577
17β-testosterone-d_2 (c)	$C_{19}H_{26}O_2d_2$		5.55	0.01	$[M+H]^+$		291.22876	291.22817	0.378
Methyltestosterone-d_3 (d)	$C_{20}H_{27}O_2d_3$		6.22	0.01	$[M+H]^+$		306.25069	306.25003	0.327
Stanozolol-d_3 (e)	$C_{21}H_{29}N_2Od_3$		6.72	0.01	$[M+H]^+$		332.27757	332.27676	0.482
Chlorotestosteron acetate-d_3 (f)	$C_{21}H_{26}ClO_3d_3$		8.22	0.01	$[M+H]^+$		368.20663	368.20574	0.679
Oestrogens									
17α-estradiol	$C_{18}H_{24}O_2$	h	5.23	0.01	$[M+H-(H_2O)]^+$		255.17434	255.17378	0.784
17β-estradiol	$C_{18}H_{24}O_2$	g	4.88	0.01	$[M+H-(H_2O)]^+$		255.17434	255.17397	0.353
Estradiol-17-acetate	$C_{20}H_{26}O_3$	h	7.85	0.01	$[M+H-C_2H_4O_2]^+$		255.17434	255.17366	0.705
Dienoestrol	$C_{18}H_{18}O_2$	h	4.35	0.02	$[M+H]^+$		267.13796	267.13735	0.487
Equilin	$C_{18}H_{20}O_2$	k	4.68	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	269.15361	269.15297	0.149
Diethylstilbestrol	$C_{18}H_{20}O_2$	j	4.94	0.01	$[M+H]^+$		269.15361	269.15294	0.706
Estrone	$C_{18}H_{22}O_2$	j	4.94	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	271.16926	271.16864	0.627
17α-ethinylestradiol	$C_{20}H_{24}O_2$	j	5.06	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$	279.17434	279.17368	0.394
α-zearalenol	$C_{18}H_{24}O_5$	j	5.14	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$, $[M+H-2(H_2O)]^+$	303.15909	303.15837	0.495
β-zearalenol	$C_{18}H_{24}O_5$	k	4.02	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$, $[M+H-2(H_2O)]^+$	303.19090	303.15847	0.429
α-zeranol	$C_{18}H_{26}O_5$	i	4.82	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$, $[M+H-2(H_2O)]^+$	305.17474	305.17410	0.524
β-zeranol	$C_{18}H_{26}O_5$	k	3.62	0.01	$[M+H-(H_2O)]^+$	$[M+H]^+$, $[M+H-2(H_2O)]^+$	305.17474	305.17402	0.492
Gestodene	$C_{21}H_{26}O_2$	j	5.27	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	311.20056	311.20034	0.964
Estradiol-benzoate	$C_{25}H_{28}O_3$	h	8.90	0.01	$[M+H]^+$		335.14813	377.21027	0.716
17β-estradiol-d_3 (g)	$C_{18}H_{21}O_2d_3$		4.88	0.01	$[M+H]^+$		277.21036	276.20331	0.688
Dienoestrol-d_2 (h)	$C_{18}H_{16}O_2d_2$		5.84	0.01	$[M+H]^+$		269.15051	269.14971	0.260
Estrone-d_4 (i)	$C_{18}H_{18}O_2d_4$		4.86	0.01	$[M+H]^+$		275.19436	275.20004	0.472
Diethylstilbestrol-d_6 (j)	$C_{18}H_{14}O_2d_6$		4.90	0.01	$[M+H]^+$		275.19127	275.20005	0.509
α-zeranol-d_4 (k)	$C_{18}H_{22}O_5d_4$		4.73	0.01	$[M+H]^+$		327.21041	327.19456	0.092
Progestins									
5α-Pregnan-3α,20β-diol	$C_{21}H_{36}O_2$	o	8.58	0.01	$[M+H-2(H_2O)]^+$		285.25768	285.25712	0.806
Norgestrel	$C_{21}H_{28}O_2$	m	6.24	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	313.21607	313.21552	0.351
Dihydroprogesterone	$C_{21}H_{32}O_2$	m	6.54	0.01	$[M+H]^+$		317.21607	317.21555	0.504
Progesterone	$C_{21}H_{30}O_2$	o	7.24	0.01	$[M+H]^+$		315.23186	315.23148	0.730
Methylprogesterone	$C_{22}H_{32}O_2$	o	8.05	0.01	$[M+H]^+$		329.24751	329.24677	0.547
17α-hydroxyprogesterone	$C_{21}H_{30}O_3$	m	5.77	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	331.22678	331.22605	0.483

Table 1 (*continued*)

Compound	Elemental formula	IS	t_R (min)	Δ t_R (min)	Diagnostic ion for quantification	Other diagnostic ions for confirmation	Theoretical mass (*m/z*)	Empirical mass (*m/z*)	Mass Accuracy (Δppm)
Megestrol	$C_{22}H_{30}O_3$	m	6.41	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	343.22678	343.22599	0.787
Medroxyprogesterone	$C_{22}H_{32}O_3$	m	6.76	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	345.24241	345.24163	0.492
17α-acetoxyprogesterone	$C_{23}H_{32}O_4$	m	6.28	0.01	$[M+H]^+$		373.23734	373.23659	0.589
Megestrol acetate	$C_{24}H_{32}O_4$	n	7.03	0.01	$[M+H]^+$		385.23734	385.23648	0.415
Medroxyprogesterone acetate	$C_{24}H_{34}O_4$	l	7.18	0.01	$[M+H]^+$		387.25299	387.25212	0.594
Flugestone acetate	$C_{23}H_{31}FO_5$	m	4.71	0.01	$[M+H]^+$	$[M+H-(H_2O)]^+$	407.22283	407.22203	0.295
Caproxyprogesterone	$C_{27}H_{40}O_4$	o	8.58	0.01	$[M+H]^+$	$[M+H-C_6H_{11}O_2-C_2H_3O]^+$	429.29994	429.29906	0.629
Progesterone-d_9 (l)	$C_{21}H_{21}O_2d_9$		7.19	0.01	$[M+H]^+$		324.28835	324.28765	0.648
Megestrol acetate-d_3 (m)	$C_{24}H_{29}O_4d_3$		7.01	0.01	$[M+H]^+$		388.25617	388.25526	0.515
Medroxyprogesterone acetate-d_3 (n)	$C_{24}H_{31}O_4d_3$		7.16	0.01	$[M+H]^+$		390.27182	390.27078	0.692
Melengestrol acetate-d_3 (o)	$C_{25}H_{29}O_4d_3$		7.27	0.01	$[M+H]^+$		400.25617	400.25540	0.550
Corticosteroids									
Prednisone	$C_{21}H_{26}O_5$	p	2.48	0.04	$[M+H-(C_2H_4O_2)-(H_2O)]^+$		299.11972	299.16353	0.100
Corticosterone	$C_{21}H_{30}O_4$	p	3.99	0.01	$[M+H]^+$		347.22169	347.22086	0.461
Cortisone	$C_{21}H_{28}O_5$	p	2.48	0.04	$[M+H]^+$		361.20095	361.19991	0.831
Prednisolone	$C_{21}H_{28}O_5$	p	2.83	0.01	$[M+H]^+$		361.20095	361.19986	0.692
Cortisol	$C_{21}H_{30}O_5$	p	2.83	0.01	$[M+H]^+$		363.21660	363.21566	0.385
Tetrahydrocortisone	$C_{21}H_{32}O_5$	p	2.42	0.04	$[M+H]^+$		365.23225	363.21566	0.275
Corticosterone acetate	$C_{23}H_{32}O_5$	p	5.17	0.02	$[M+H]^+$		389.23225	389.23121	0.668
Dexamethasone	$C_{22}H_{29}FO_5$	q	3.80	0.03	$[M+H]^+$		393.20718	393.20641	0.636
Prednisolone acetate	$C_{23}H_{30}O_6$	p	4.09	0.01	$[M+H]^+$		403.21152	403.21067	0.273
Cortisone acetate	$C_{23}H_{30}O_6$	p	9.05	0.01	$[M+H]^+$		403.21152	403.21057	0.942
Hydrocortisone 21-acetate	$C_{23}H_{32}O_6$	p	4.04	0.01	$[M+H]^+$		405.22717	405.22625	0.642
Prednisone-d_8 (p)	$C_{21}H_{18}O_5d_8$		2.01	0.01	$[M+H]^+$		367.23551	367.23534	0.463
Dexamethasone-d_4 (q)	$C_{22}H_{25}FO_5d_4$		3.76	0.01	$[M+H]^+$		397.23229	397.23143	0.680

optimal MS parameters of the Q-Exactive™ were S-lens RF-level 70, full-scan events and operated in polarity switching mode. Both scans were performed with a resolution of 70 000 FWHM (Full Width at Half Maximum) at 1 Hz (1 scan per sec) and scan ranges from 60 to 900 Da. Furthermore, the scans were applied by targeting the automatic gain control (AGC) at ultimate mass accuracy (1×10^5 ions) and a maximum injection time of 50 ms. Initial instrument calibration was carried out by infusing calibration mixtures for the positive and negative ion mode (LTQ Velos ESI positive and negative ion calibration solution, Thermo Fisher Scientific, San Francisco, USA). Instrument control and data processing were carried out by Xcalibur 4.0 software (Thermo Fisher Scientific, San Francisco, USA).

2.3. Sample preparation and extraction

2.3.1. Statistical experimental designs for the optimization

A three-step statistical workflow, based on experimental designs, was used to efficiently optimize sample preparation and extraction [32]. For this purpose, reference seawater [31] was used, which was spiked with different amounts of EDCs, according to the optimization stage, i.e. to reach 50 ng L^{-1} for screening (27 experiments); 10 ng L^{-1} for eluent optimization (11 experiments) and 5 ng L^{-1} for response surface modelling (15 experiments).

During the screening phase, 13 parameters that could affect the EDC extraction efficiency were selected based on literature [16,33–35] and investigated for their effects (Table A.2..). In case of significance, they were retained for further optimization. The significance of these selected variables was determined by using a three-level fractional factorial resolution IV experimental design. A second step entailed the optimization of the extraction solvent using a simplex lattice mixture design for three variables (the percentage of methanol, acetonitrile and water). A third step consisted of optimizing the selected significant variables through response surface modelling (RSM), using a central composited faced-centered (CCF) design.

The software program JMP 12.0 (SAS Institute Inc, Cary, USA) was used to select, evaluate, and model the appropriate statistical experimental designs. All models were optimised using the summarised normalised area, which was selected to take into account the high number of analytes and ensure equal compound contribution. Responses were statistically evaluated by one-way analysis of variance (ANOVA) at a confidence interval of 95% (p-value of 0.05). Finally, the optimised extraction settings that yielded the highest response were calculated by using a generalised reduced gradient non-linear algorithm and RSM.

2.3.2. Final protocol for EDC extraction

2.5 L grab samples were filtered (Glass Microfibre Filters Whatman™, 0.45 μm, 90 × 90 mm), acidified with 1 M HCl and stored in dark amber glass bottles at 4 °C. Upon extraction, samples were brought to room temperature by vibrating. Thereafter, the pH was adjusted to 7 using 1 M NaOH and a mixture of deuterated internal standards was spiked (n = 17, 25 μL of 10 ng μL^{-1}) to the grab samples. Subsequently, the H_2O-phillic DVB sorbents were conditioned with 20 mL of 5% acetonitrile and 20 mL of ultrapure water under vacuum. Next, the samples were drawn through the H_2O-phillic DVB Speedisks under vacuum, followed by a washing step with 20 mL of ultrapure water, upon which a vacuum was applied on the speedisks to remove residual water drops. Afterwards, elution was performed by gravity using sequential 5 mL of pure acetonitrile and 5 mL of acetonitrile, with the latter being acidified with 0.1% formic acid. The combined extracts were vaporized in the Turbovap under a gentle stream of nitrogen at a temperature of 50 °C until dry. After this, the extract was reconstituted in 150 μL of methanol and ultrapure water (40/60, v/v), centrifuged at 2430 g, and the supernatant was transferred into LC-MS glass vial prior to analysis.

2.4. Method validation

The optimised analytical method was validated on reference

seawater in order to evaluate its fitness-for-purpose. Currently, no specific criteria for the validation of methods for analysis of micropollutants in the marine environment are available. The only European guideline that is currently available for analytical evaluation of the water status is CD 2009/90/EC [26], in which it is stated that the variation coefficient of the reported concentration must be below 50%. Furthermore, it stipulates that the detection limit has to be 30% below the environmental quality standard, which is defined by the degree of concentration of a substance that water should not exceed to maintain the environmental quality objective. Currently, no environmental quality standards are available concerning the occurrence of steroidal EDCs in the marine environment. Therefore, stricter guidelines were consulted for additional performance criteria in analytical method validation, i.e. CD 2002/657/EC [25], Eurachem guidelines [27] and review articles [28,29].

Evaluation criteria included the empirical method detection (MDL) and quantification limit (MQL), linearity, specificity and selectivity, trueness, and precision. The MDL, MQL and the linearity were investigated by constructing three times a 13-point matrix-matched calibration curve (0, 0.125, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 10, 20, 30, 40 and 50 ng L^{-1}). Furthermore, the specificity, selectivity, trueness and precision were examined by spiking the seawater at 1.5, 2.0 and 2.5 times the MQL-level in sixfold. This procedure was repeated on three different days by two different operators. In addition, also 20 blanks, i.e. non-spiked reference seawater, were analysed.

In parallel, a cross-validation on fresh tap water was performed to assess the matrix-versatility of the presented method. During this cross-validation, a 13-point matrix-matched calibration curve was constructed twice to determine the linearity performance, while the specificity, selectivity, trueness and precision were investigated by enriching the samples with 1.5 times the MQL-level (n = 18).

2.5. Application of the analytical method to real samples

The suitability of the method was evaluated by applying it on grab samples, collected at four different locations, i.e. 51.22263°, 2.9357°; 51.340073°, 3.203393°; 51.24683°, 3.113615°; and 51.360494°, 3.113615°, in the Belgian Part of the North Sea (BPNS) during two different periods of the year, i.e. fall 2016 (November 25th) and winter 2017 (February 2nd). More specifically, 2.5 L grab samples were taken at a depth of 3 m, using Niskin bottles [36].

Upon arrival in the lab, samples were acidified to pH 3 using 1 M HCl and stored in dark amber glass bottles at 4 °C prior to extraction. Acidification did not significantly impact the recovery of EDCs during extraction optimization. During method optimization, it had been verified that this sample acidification did not affect the

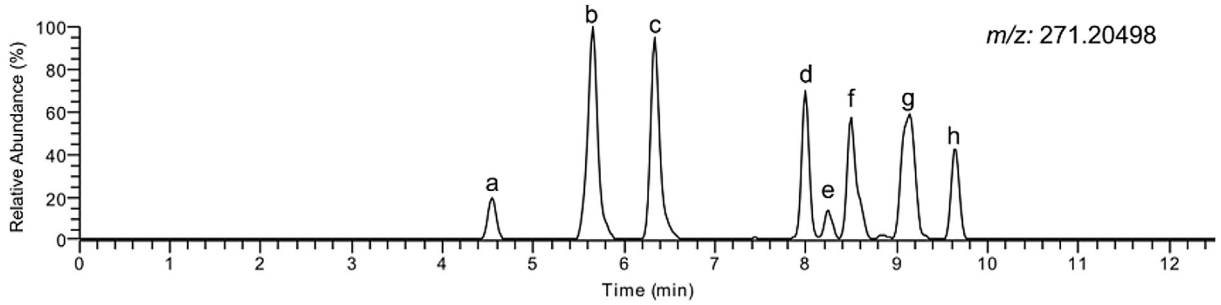

Fig. 1. Chromatographic separation of all EDCs with an *m/z* of 271.20498 Da (mass tolerance = 3 ppm), depicting (a) 17β-trenbolone, (b) 17α-trenbolone, (C) 11β-hydroxyandrosterone, (d) testosterone acetate, (e) chlorotestosterone acetate, (f) caproxyprogesterone, (g) testosterone phenylpropionate and (h) testosterone 17β-cypionate.

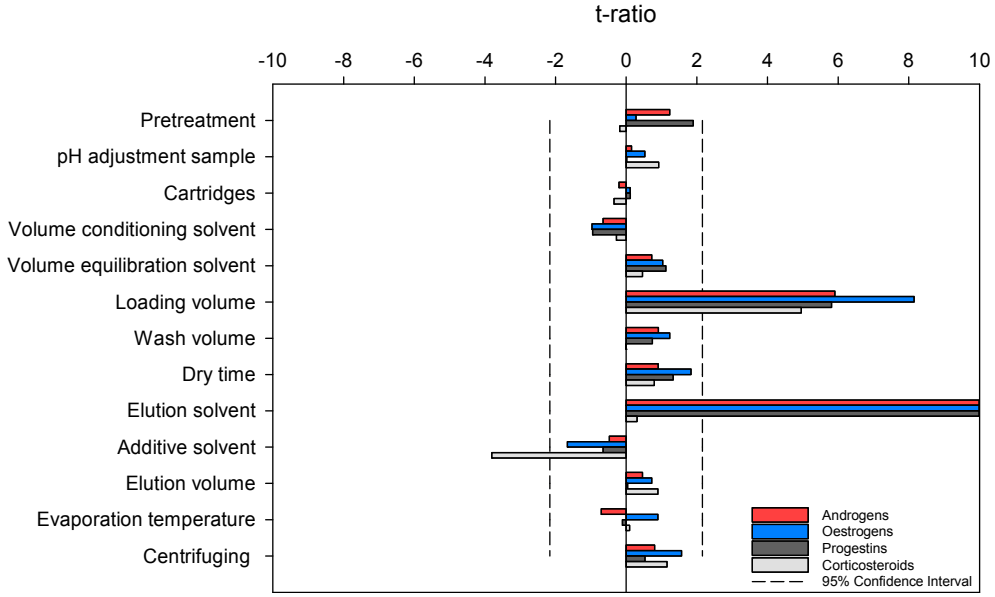

Fig. 2. T-ratio effect diagram, illustrating the significance of different extraction parameters for the 4 EDC classes. T-ratio effect bars crossing the 95% confidence interval (dashed line) indicate a significant effect of the respective parameter in the extraction process.

Table 2
Summary of the method validation performance characteristics as determined for EDCs in seawater.

Analyte	Recovery (%)	MDL (ng L^{-1})	MQL (ng L^{-1})	Repeatability RSD (%) (n = 18)	Within-laboratory reproducibility RSD (%) (n = 12)	R^2	Best IS	Other suitable IS
Androgens								
Methandriol	101.3 ± 6.3	0.06	0.50	6.2 ± 3.0	3.8 ± 1.9	0.9980	e	
17α-trenbolone	100.0 ± 7.2	0.25	0.50	5.5 ± 1.5	6.8 ± 2.8	0.9993	b	m, n
17β-trenbolone	101.6 ± 6.3	0.25	0.50	5.5 ± 1.5	6.8 ± 2.8	0.9949	b	m, n
11β-hydroxyandrosterone	102.5 ± 9.3	0.25	0.50	7.5 ± 4.9	7.9 ± 5.6	0.9949	c	b, f, o
Testosterone 17β-cypionate	108.3 ± 8.4	0.13	0.50	5.8 ± 1.2	8.7 ± 3.2	0.9981	d	b, c, e, f, h
17β-dihydroandrosterone	97.1 ± 6.2	0.50	0.13	6.2 ± 0.8	6.7 ± 0.7	0.9903	e	
Androsterone	100.8 ± 4.8	0.25	0.25	3.7 ± 1.3	4.3 ± 1.4	0.9968	e	a, b, f, m, o, q
19-nortestosterone	97.4 ± 5.6	0.13	0.75	4.8 ± 1.1	6.3 ± 2.5	0.9984	b	c, d, e, h, m
1,4-Androstadienedione	97.4 ± 7.8	0.06	0.25	7.9 ± 0.6	7.1 ± 5.4	0.9987	b	c, d, f, h, l, m, n, o
11-ketoetiocholanolone	98.3 ± 7.8	0.13	0.25	7.0 ± 4.0	7.9 ± 5.3	0.9985	b	i, j
Androstenedione	97.4 ± 5.7	0.13	0.50	4.3 ± 1.5	5.7 ± 0.8	0.9995	b	c, d, f, l, m, n, o, q
Mestanolone	99.8 ± 5.8	0.25	0.75	5.0 ± 0.8	6.6 ± 1.3	0.9965	e	i, j
17α-testosterone	100.3 ± 6.4	0.13	0.25	5.4 ± 2.0	6.3 ± 1.9	0.9975	d	c, e, f, h, l, m, n, o
17β-testosterone	98.9 ± 8.2	0.06	0.25	5.8 ± 3.0	9.6 ± 4.2	0.9998	c	d, e, f, h, l, m, n, o
5α-dihydrotestosterone	98.2 ± 6.3	0.25	0.13	6.4 ± 0.6	6.6 ± 0.7	0.9923	e	
19-Norethindron	98.1 ± 6.3	0.50	1.00	5.0 ± 2.3	7.2 ± 2.7	0.9975	a	i, j
Methylboldenone	100.5 ± 7.9	0.25	1.00	6.9 ± 2.5	7.7 ± 2.6	0.9985	a	b, f
11-ketotestosterone	99.6 ± 8.2	0.13	0.25	6.2 ± 2.9	6.9 ± 3.1	0.9974	b	e
Formestane	100.6 ± 8.6	0.13	0.25	7.8 ± 1.8	8.7 ± 3.1	0.9965	b	c, d, f, h, l, n, o
Norethandrolone	101.8 ± 6.9	0.06	0.13	5.8 ± 2.2	6.8 ± 2.7	0.9962	e	d, l
Methyltestosterone	98.8 ± 5.7	0.13	0.25	4.8 ± 1.2	6.0 ± 2.3	0.9992	d	c, d, e, f, g, h, l, m, n, o
Trenbolone acetate	100.6 ± 5.8	0.06	0.50	4.7 ± 1.2	5.9 ± 3.1	0.9951	e	a
Ethynyl testosterone	103.3 ± 6.9	0.06	0.25	5.5 ± 2.7	6.0 ± 3.2	0.9987	d	a, b, c, d, e, f, g, h, l, m, n, o, p, q
Stanozolol	98.9 ± 6.6	1.00	1.00	5.3 ± 2.8	8.0 ± 5.7	0.9977	e	b, c, d, f, g, h, l, m, n, p
Testosterone acetate	100.6 ± 6.1	0.06	0.75	4.7 ± 1.4	7.6 ± 2.8	0.9983	f	l
Fluoxymesterone	102.0 ± 6.3	2.50	5.00	5.0 ± 2.0	6.5 ± 1.3	0.9975	b	c, d, g, j, o
Testosterone propionate	100.4 ± 6.4	0.13	0.25	5.1 ± 1.8	7.2 ± 2.4	0.9973	f	b, l, n, o
Chlorotestosteron acetate	100.6 ± 5.6	0.50	0.50	3.9 ± 0.7	7.1 ± 3.0	0.9962	f	a, e
Testosterone benzoate	102.9 ± 6.7	0.50	0.75	4.9 ± 1.9	8.4 ± 3.1	0.9978	f	c, d, l, m
Testosterone phenylpropionate	100.8 ± 6.4	0.25	0.75	5.4 ± 2.2	7.0 ± 2.3	0.9952	f	a, b, c, k, l, n, o, p, q
19-nortestosterone-17-decanoate	102.5 ± 6.7	2.50	2.50	5.4 ± 1.9	7.6 ± 2.3	0.9926	f	h
Oestrogens								
17α-estradiol	101.6 ± 7.8	0.25	5.00	7.0 ± 3.1	7.1 ± 3.1	0.9976	h	a, c, d, i, l, n, o, q
17β-estradiol	100.9 ± 8.4	0.06	2.50	7.0 ± 2.8	8.9 ± 4.6	0.9959	g	a, c, d, h, i, l, n, o, q
Estradiol-17-acetate	100.4 ± 7.8	0.06	0.75	6.7 ± 3.6	8.0 ± 5.3	0.9937	h	c, d, f, l, m, q
Dienoestrol	100.3 ± 7.4	0.25	5.00	6.3 ± 2.9	8.4 ± 5.0	0.9964	h	
Equilin	102.0 ± 7.6	0.13	0.25	6.4 ± 3.2	7.8 ± 4.3	0.9950	k	b, f, m, o
Diethylstilbestrol	101.8 ± 8.3	0.25	0.25	6.2 ± 2.7	10.0 ± 5.7	0.9958	j	b, c, d, f, m, o
Estrone	102.7 ± 8.0	0.06	0.25	6.7 ± 1.8	8.6 ± 3.2	0.9992	j	c, d, n, o
17α-ethinylestradiol	102.8 ± 6.2	2.50	5.00	4.7 ± 1.9	7.8 ± 4.2	0.9958	j	i
a-zearalenol	101.1 ± 6.0	1.00	2.50	6.5 ± 2.7	8.0 ± 4.3	0.9921	j	f
b-zearalenol	101.4 ± 2.9	0.13	0.75	6.0 ± 2.8	7.6 ± 3.7	0.9931	k	h
a-zeranol	99.9 ± 9.0	0.13	0.75	8.1 ± 2.5	9.7 ± 3.9	0.9947	i	b, d, g, j, l, m, n
b-zeranol	101.4 ± 7.3	0.13	0.75	6.6 ± 1.8	7.0 ± 2.5	0.9908	k	b, d, g, j, l, m, n
Gestodene	103.4 ± 6.6	0.25	0.50	5.1 ± 1.9	7.0 ± 3.5	0.9946	j	b, c, d, e, h, l, m, n, p, q
Estradiol-benzoate	101.2 ± 6.1	1.00	2.50	4.9 ± 0.4	6.9 ± 2.2	0.9941	h	e, l, m
Progestins								
5α-Pregnan-3α,20β-diol	101.3 ± 1.2	2.50	2.50	4.0 ± 1.2	5.8 ± 1.3	0.9917	o	
Norgestrel	101.0 ± 7.1	0.06	0.25	5.3 ± 1.0	6.8 ± 1.2	0.9949	m	a, b, c, d, e, f, g, h, l, m, n, q
Dihydroprogesterone	98.3 ± 8.0	0.06	0.25	6.4 ± 1.1	8.1 ± 2.0	0.9973	m	b, c, d, f, h, i, j, l, n, o
Progesterone	99.0 ± 8.4	0.06	0.50	6.7 ± 4.4	8.8 ± 5.0	0.9984	o	b, c, l, n
Methylprogesterone	102.2 ± 6.2	0.06	0.25	5.5 ± 1.7	5.9 ± 2.1	0.9961	o	a, b, c, d, e, f, h, l, m, n, p
17α-hydroxyprogesterone	100.1 ± 6.9	0.13	0.25	5.7 ± 3.1	6.3 ± 4.1	0.9986	m	b, c, d, f, h, l, n, o
Megestrol	101.9 ± 7.7	0.75	1.00	5.7 ± 2.1	8.8 ± 3.2	0.9966	m	b, c, d, f, h, j, l, n, o
Medroxyprogesterone	101.2 ± 4.5	0.13	0.50	4.1 ± 0.6	4.7 ± 1.1	0.9966	m	b, g, n
17α-acetoxyprogesterone	102.5 ± 5.1	0.13	0.50	4.0 ± 0.6	6.4 ± 2.7	0.9952	m	a, b, c, d, f, l, n, o, q
Megestrol acetate	101.7 ± 5.3	0.50	0.75	4.4 ± 1.2	4.7 ± 1.3	0.9976	n	b, d
Medroxyprogesterone acetate	101.5 ± 3.9	0.50	1.00	3.5 ± 0.4	4.0 ± 0.7	0.9976	l	i, j
Flugestone acetate	102.5 ± 7.2	0.75	1.00	6.2 ± 2.6	6.7 ± 3.1	0.9969	m	b, c, d, e
Caproxyprogesterone	102.0 ± 6.5	0.25	0.75	4.4 ± 0.2	8.5 ± 2.1	0.9977	o	b, c, d, e, f, l, m, n
Corticosteroids								
Prednisone	103.4 ± 7.8	0.25	0.50	6.0 ± 1.8	7.9 ± 1.9	0.9914	p	b, c, d, e, h, m, n
Corticosterone	102.0 ± 5.7	0.50	2.50	5.8 ± 2.4	7.4 ± 3.1	0.9909	p	e
Cortisone	101.7 ± 7.5	0.13	2.50	7.5 ± 3.8	10.5 ± 3.2	0.9952	p	e, l, m
Prednisolone	102.8 ± 9.1	0.13	2.50	7.0 ± 2.7	8.2 ± 1.6	0.9985	p	
Cortisol	102.3 ± 8.4	0.25	0.75	8.3 ± 5.0	10.0 ± 3.9	0.9926	p	c, i, j
Tetrahydrocortisone	102.5 ± 9.7	0.25	5.00	6.5 ± 2.2	9.3 ± 3.1	0.9985	p	
Corticosterone acetate	103.0 ± 5.7	0.50	2.50	4.2 ± 1.5	5.5 ± 2.6	0.9952	p	a, b, c, d, f, I, j, o

(*continued on next page*)

Table 2 (*continued*)

Analyte	Recovery (%)	MDL (ng L^{-1})	MQL (ng L^{-1})	Repeatability RSD (%) (n = 18)	Within-laboratory reproducibility RSD (%) (n = 12)	R^2	Best IS	Other suitable IS
Dexamethasone	103.0 ± 8.5	2.50	2.50	4.2 ± 1.5	5.5 ± 2.6	0.9952	q	
Prednisolone acetate	99.9 ± 6.9	5.00	5.00	5.8 ± 1.4	7.1 ± 2.0	0.9906	p	
Cortisone acetate	101.3 ± 3.2	2.50	2.50	7.8 ± 3.1	7.6 ± 3.8	0.9952	p	
Hydrocortisone 21-acetate	100.0 ± 8.5	5.00	5.00	6.7 ± 1.8	8.7 ± 4.8	0.9984	p	a, c

recovery of EDCs. Additionally, amber glass bottles were used as storage device. Previous research demonstrated that glass is the best material for storage of aqueous samples for EDC analysis since loss of hydrophobic EDCs was limited to 1% or less [37,38].

3. Results and discussion

3.1. Method development

3.1.1. Liquid chromatography

Given the superior performance of UHPLC in terms of chromatographic resolution compared to conventional HPLC [39], the UHPLC separation strategy was selected in this work for multi-EDC profiling. Optimal conditions, relating to the stationary phase, flow rate, mobile phase composition, additives, column temperature, and injection volume, were determined by studying their impacts on the inter-linked resolution, chromatographic peak shape, and interfering background for the 70 targeted analytes. Optimal UHPLC conditions have been reported earlier (section 2.2.).

Optimization of the UHPLC parameters enabled the separation of 70 target steroidal EDCs (Figures A.1. - A.2), covering a broad polarity range (log P ranging from 0.5 to 7.9, Table A.1.) with retention times ranging from 2.1 to 9.9 min (Table 1). In addition, chiral isomers and mass analogues (having overlapping mass extraction windows) were baseline separated, except ethylestrenol and 17β-dihydroandrosterone were separated below the 10% valley rule. This is exemplified in Fig. 1, where 8 compounds with an accurate empirical mass of 271.20498 Da and 3 ppm mass tolerance were successfully chromatographically separated (Fig. 1), including the chiral compounds 17 α- and β-trenbolone, as well as the mass analogues 11β-hydroxyandrosterone, testosterone acetate, chlorotestosterone acetate, caproxyprogesterone, testosterone phenylpropionate and testosterone 17β-cypionate. Compared to relevant literature [18–20], the overall UHPLC conditions were found to be high throughput for the simultaneous separation of 70 target steroidal EDCs in a single injection with a total run time of 12.5 min.

3.1.2. Mass spectrometry

To enable a most reliable and accurate quantification of EDCs, the ionization and mass spectrometric parameters were optimised on seawater extracts. For optimisation of the APCI ionization parameters (reported in section 2.2) the overall peak intensity of the EDCs was the main evaluation criterion. The EDC mass spectra were mainly characterized by the presence of the positive pseudomolecular ion $[M+H]^+$ and or dehydrated positive pseudomolecular ion $[M + H\text{-}nH_2O]^+$ with n variating between 1 and 2, which has been reported earlier for steroids [40]. The most abundant diagnostic ion was selected for identification and quantitation (Table 1). The other diagnostic ions were used as an additional confirmation tool backing the isotopic signature of the target compounds, ^{13}C-isotope. The remaining mass spectrometric parameters that were optimized, comprised the resolving power and AGC target. The resolving power was determined by a trade-off between the achievable mass accuracy and the number of data points across the chromatographic peak. On the one hand, an improved mass accuracy (obtained by a higher resolving power) resulted in a better selectivity and thus exclusion of isobaric matrix interferences, which contributes towards unequivocal identification and accurate quantitation. On the other hand, an increasing resolving power was accompanied by less data points across the peak, which resulted in a lack of sensitivity and repeatability. Therefore, a resolving power of 70,000 FWHM was selected since it offered a compromise between high mass accuracy (mass deviations < 3 ppm) and sufficient data points across the chromatographic peaks (>10) [41]. Furthermore, the optimal AGC target was set to $1e^5$ ions, as this setting displayed the lowest mass deviation (<3 ppm) at the MQL-level.

3.1.3. Extraction procedure

A three-level fractional resolution IV experimental design was used for screening, thereby assessing the effect of 13 parameters on the extraction efficiency (Fig. 2). Ten parameters turned out to be non-significant (p-value > 0.05) for all EDC classes, whereas the remaining three parameters were observed to be significant (p-value < 0.05) for at least one of the classes. Loading volume was found significant for all classes whereas the solid phase extraction (SPE) elution steps were significant for some classes. More specifically, the corticosteroids' summarised normalised area was significantly affected by the solvent additive, whereas this was not the case for the elution solvent. However, the elution solvent had a significant effect on the summarised normalised area of the androgens, progestins, and oestrogens. Based on these findings and the different elution solvents reported in literature [16,33–35], the elution solvent was further optimized using a simplex lattice mixture design, which pointed towards the use of 100% acetonitrile instead of a water-methanol-acetonitrile mixture. The better results that were obtained for acetonitrile are in line with literature, because acetonitrile enables EDCS that are tightly adsorbed to the sorbent phase to undergo lower surface tension/interaction with the sorbent, as such facilitating elution [42].

In a last step, the loading volume and solvent additive were optimised using RSM. Hereby, the RSMs suggested maximal loading volumes. Nevertheless, because of clogging of the sorbent phase at this high loading volume, it was technically not possibly to exceed 2.5 L. Optimization of the solvent additive demonstrated a better overall sensitivity for the corticosteroids in the presence of 0.1% formic acid, whereas acid-free solvents favoured the recovery of the androgens, oestrogens and progestins. The use of formic acid is assumed to slightly increase the polarity of acetonitrile, which may result in a better elution of the more polar compounds including the corticosteroids. Therefore, to efficiently elute the representatives of all EDC classes from the sorbent phase, a sequential use of two elution solvents was implemented; i.e. pure and acidified (0.1% formic acid) acetonitrile.

3.2. Method validation

The goal of this work was to develop an analytical methodology that allows the simultaneous quantification of 70 different EDC residues in the marine environment. To ensure accurate

Table 3
Comparison of our newly developed method with methods from literature for measurement of steroidal EDCs in different aqueous samples.

	This study		Zhang et al. 2014 [15]		Torres et al. 2015 [17]		Petrie et al. 2016 [18]		Fayad et al. 2013 [19]		Goh et al. 2016 [20]		Anumol et al. 2015 [21]	
Method	SPE-UHPLC-HRMS		SPE-HPLC-MS/MS		SPE-LC-MS/MS		SPE-UPLC-MS/MS		Online-SPE-LC-MS/MS		Online-SPE-LC-MS/MS		Online-SPE-LC-MS/MS	
Matrix	Seawater		Seawater		Surface and drinking water		River water		Wastewater (effluent)		Wastewater		Ultrapure water	
Chromatographic column	Hypersil Gold (100 mm × 2.1 mm; 1.9 μm)		Thermo Scientific (100 mm × 3 mm; 3.0 μm)		Zorbax Eclipse Plus (100 mm × 3.0 mm; 3.5 μm)		BEH C18 (150 mm × 1.0 mm; 1.7 μm)		Hypersil Gold (100 mm × 2.1 mm; 1.9 μm)		Accucore (100 mm × 4.6 mm; 2.6 μm)		Agilent Poreshell (50 mm × 2.1 mm; 2.7 μm)	
Ionization	APCI		ESI		ESI		ESI		API		APCI		ESI	
MS device	Q-Exactive Benchtop		MS/MS Agilent Technology		Agilent 6410 triple Quad MS		Xevo TQD triple quadrupole		Quantum Ultra AM triple quadrupole		API 4000™ MS/MS		Agilent 6410 triple Quad MS	
Analysis time (min)	12.5		10		5		22.5		14		17		14.5	
Storage device	Amber glass bottles		Not specified		Amber glass bottles		Amber glass bottles		Amber glass bottles		Amber glass bottles		Amber glass bottles	
Filtration	0.45 μm Whatmann glass filter		0.7 μm GF/F		0.47 μm glass filter		0.7 μm GF/F		0.3 μm glass filter		0.2 μm nylon filter		0.2 μm Captiva PES filter	
SPE sorbent phase	H_2O-phillic DVB		Oasis HLB		Oasis HLB		Oasis MCX		Oasis HLB		Oasis HLB		PLRP	
Sample volume (L)	2.5		8		0.2		0.05		0.01		0.0025		0.0017	
Flow rate (mL min^{-1})	75		Not specified		4		5		1.5		0.5–1.0		1	
Elution solvent	CH_3CN, 0.1% CH_2O_2 in CH_3CH		CH_3OH		CH_3OH		0.6% $C_2H_2O_2$ and 7% NH_4OH in CH_3OH		CH_3OH		0.1% NH_4OH in CH_3CN		CH_3CN	
Steroidal compounds	70		4		5		3		8		9		2	
Analytes	MDL (ng L^{-1})	MQL (ng L^{-1})	MDL (ng L^{-1})	MQL (ng L^{-1})	MDL (ng L^{-1})	MQL (ng L^{-1})	MDL (ng L^{-1})	MQL (ng L^{-1})	MDL (ng L^{-1})	MQL (ng L^{-1})	MDL (ng L^{-1})	MQL (ng L^{-1})	MDL (ng L^{-1})	MQL (ng L^{-1})
17β-testosterone	0.06	0.25			0.5	5							2.5	4.4
19-Norethindrone	0.50	1.00							34	35				
17β-estradiol	0.06	2.5	0.1		0.7	7	0.9	4.48	21	36				
Equilin	0.13	0.25									0.44	1.44		
Diethylstilbestrol	0.25	0.25									0.97	3.7		
Estrone	0.06	0.25	0.05		0.5	5	0.78	3.92	16	30	0.16	0.42		
17α-ethinylestradiol	2.50	5.00	0.3		0.7	7	0.98	4.91	18	33	0.6	2.6		
Levonorgestrel	0.06	0.25							18	50			10	11.6
Progesterone	0.06	0.25			0.3	3			8	21				
Cortisone	0.13	2.50									0.48	1.04		
Dexamethasone	2.50	2.50									0.22	0.61		

quantification, the analytical method was validated, whereby data on the MDL and MQL, specificity, selectivity, linearity, trueness, and precision were generated.

3.2.1. Limits of detection and quantification (MDL and MQL)

The detection and quantification of EDC residues using HRMS presents new challenges to the determination of MDLs and MQLs, as traditionally estimated by theoretical or empirical calculations based on signal-to-noise ratios. The signal-to-noise ratios obtained by HRMS are mainly of infinite magnitudes, resulting in virtually low detection and quantification limits. To deal with these virtual estimations, new strategies are required based on more practical criteria. Therefore, the validation criteria stated in CD 2002/657/EC (food safety), CD 2009/90/EC (water monitoring) and Eurachem 2016 (general guidelines) - for measuring residues in the aquatic environment - were combined and refined as was previously described by Vergeynst et al. [43], but with usage of an additional criterion, i.e. identity confirmation through the ^{13}C-isotope and the $^{13}C/^{12}C$-ratio of each target compound at the corresponding theoretical MDL.

The MQLs for the androgens, oestrogens, progestins and corticosteroids ranged from, respectively, 0.13–5.00 ng L^{-1}, 0.25–5.00 ng L^{-1}, 0.25–2.50 ng L^{-1} and 0.50–5.00 ng L^{-1}, whereas the MDLs for all classes ranged from 0.06 to 2.50 ng L^{-1}. Our empirical limits, i.e. MDLs and MQLs in seawater (Table 2), are comparable or even lower than the theoretical limits achieved in previous studies, using UHPLC-MS/MS (Table 3).

3.2.2. Selectivity

The specificity and selectivity were evaluated by analysing blank samples as well as samples spiked at 1.5 times the MQL (Table A.3.). As true blanks, reference seawater was used, containing no measurable residues of exogenous EDCs at their accurate mass and specific retention time. A significant increase was observed at the accurate mass and specific retention time when EDCs were added to the blank samples, taking into account a maximal RSD of 20%. The above-mentioned observations confirmed that the optimised method was selective for the 70 target EDCs in the presence of other matrix constituents. Identification was based on accurate mass and relative retention time, i.e. the ratio between retention time of the analyte and its deuterated internal standard, which ensured the high selectivity of the method. In addition, the mass deviation (<1 ppm) and retention time deviation (<0.05 min) confirmed the instrumental stability (n = 110, time period = 3 days) of the developed UHPLC-HRMS method (Table 1).

3.2.3. Linearity and deuterated internal standards

Linearity was evaluated by setting up 13-point matrix-matched calibration curves in triplicate, with concentration levels ranging from 0 to 50 ng L^{-1} for the compounds of interest. The linearity was analysed by establishing weighted linear regression models. These regression models (Table A.4.) indicated good linearity ($R^2 \geq 0.99$) and no lack of fit (95% confidence interval; F-test, $p > 0.05$) [44]. During the evaluation of the linearity performance, appropriate deuterated internal standards were determined for each compound (Table 2), thereby pursuing a RSD for the peak area ratio ≤ 20% and a good linearity ($R^2 > 0.99$ and no lack of fit).

3.2.4. Trueness and precision

Trueness and precision were assessed at different levels, which

Table 4
Detailed data of the grab samples taken at 4 different locations in the BPNS and 2 different time points (for each time point and each location investigated in fourfold, n = 4).

Grabsamples	Analytical Limits		Sampling Campaign Fall (2016)				Sampling Campaign Winter (2017)		
	MDL (ng L^{-1})	MQL (ng L^{-1})	51.2263°, 2.9357°	51.2468°, 3.1136°	51.3401°, 3.2003°	51,3605°, 3.1136°	51.2263°, 2.9357°	51.2468°, 3.1136°	51.3401°, 3.2003°
Androgens									
17β-trenbolone	0.25	0.50					0.91 ± 0.47	< MQL	0.57 ± 0.19
11β-hydroxyandrosterone	0.25	0.50		< MQL	0.63 ± 0.08				
Testosterone cypionate	0.13	0.50			1.31 ± 0.35				
Androsterone	0.25	0.25	3.63 ± 0.08	1.61 ± 0.74	3.33 ± 1.43	2.57 ± 0.08		4.41 ± 0.06	
19-nortestosterone	0.13	0.75					< MQL		< MQL
1,4-Androstadienedione	0.06	0.25	1.62 ± 0.30	1.16 ± 0.14	1.48 ± 0.52	1.15 ± 0.30	0.83 ± 0.69	< MQL	0.28 ± 0.07
Androstenedione	0.13	0.50					< MQL	< MQL	< MQL
Mestanolone	0.25	0.75			< MQL			3.34 ± 0.02	3.37 ± 0.01
17α-testosterone	0.13	0.25	1.10 ± 0.26		0.91 ± 0.30	0.51 ± 0.26	0.35 ± 0.07	0.34 ± 0.08	0.26 ± 0.02
5α-dihydrotestosterone			1.89 ± 0.52	0.50 ± 0.17	0.85 ± 0.23	0.52 ± 0.52		3.79 ± 0.06	3.79 ± 0.03
11-ketotestosterone	0.13	0.25		1.44 ± 0.10	2.06 ± 0.56		2.31 ± 0.28	0.46 ± 0.03	0.52 ± 0.14
Formestane	0.13	0.25					2.04 ± 0.61	0.83 ± 0.24	0.69 ± 0.09
Methyltestosterone	0.13	0.25		< MQL	0.30 ± 0.07				
Ethynyl testosterone	0.06	0.25	0.65 ± 0.05						0.32 ± 0.01
Testosterone acetate	0.06	0.75	1.12 ± 0.07	0.95 ± 0.03	1.01 ± 0.05	0.96 ± 0.07	< MQL	< MQL	< MQL
Testosterone propionate	0.13	0.25	2.69 ± 1.13	1.24 ± 0.09	1.31 ± 0.12	1.26 ± 1.13		0.48 ± 0.01	0.50 ± 0.02
Testosterone benzoate	0.50	0.75			1.51 ± 0.24				
Oestrogens									
17α-estradiol	0.25	5.00					< MQL	< MQL	< MQL
17β-estradiol	0.06	2.50	9.69 ± 3.96	6.72 ± 0.90	6.40 ± 0.43	6.83 ± 3.96	6.37 ± 0.09		7.62 ± 0.76
Estradiol-17-acetate	0.06	0.75	10.39 ± 6.89	2.83 ± 1.30	3.04 ± 1.71	2.51 ± 6.89		1.49 ± 0.32	2.00 ± 0.73
Dienoestrol	0.25	5.00						< MQL	< MQL
Estrone	0.06	0.25					1.93 ± 0.21	1.90 ± 0.30	1.99 ± 0.46
α-zeranol	0.13	0.75					3.85 ± 1.08	3.14 ± 0.42	
Gestodene	0.25	0.50							1.31 ± 0.02
Estradiol-benzoate	1.00	2.50			3.54 ± 0.18				
Progestins									
Norgestrel	0.06	0.25			0.39 ± 0.00				1.73 ± 0.01
Dihydroprogesterone	0.06	0.25						1.92 ± 0.01	1.92 ± 0.01
Progesterone	0.06	0.50	< MQL		< MQL	< MQL		0.81 ± 0.03	0.73 ± 0.06
Methylprogesterone	0.06	0.25	0.75 ± 0.54		< MQL	< MQL	0.66 ± 0.01	0.65 ± 0.02	0.67 ± 0.03
17α-hydroxyprogesterone	0.13	0.25		0.75 ± 0.10				1.78 ± 0.00	1.79 ± 0.02
Megestrol	0.75	1.00						2.64 ± 0.01	
Medroxyprogesterone	0.13	0.50	0.84 ± 0.33	< MQL	0.65 ± 0.19	0.59 ± 0.33	0.66 ± 0.01	0.65 ± 0.02	0.67 ± 0.03
17α-acetoxyprogesterone	0.13	0.50	1.20 ± 0.11						2.10 ± 0.00
Megestrol acetate	0.50	0.75	< MQL	< MQL	< MQL	< MQL			
Medroxyprogesterone acetate	0.50	1.00	1.14 ± 0.96	< MQL	< MQL	< MQL			
Caproxyprogesterone	0.25	0.75					1.12 ± 0.23		0.89 ± 0.02
Corticosteroids									
Prednisone	0.25	0.50	39.14 ± 8.90	13.09 ± 3.49		9.17 ± 8.90			
Corticosterone	0.50	2.50	4.56 ± 1.73	2.48 ± 0.47	2.59 ± 0.47	3.14 ± 1.73		< MQL	< MQL
Cortisone	0.13	2.50	28.18 ± 17.87	4.79 ± 1.72	6.96 ± 3.23	10.02 ± 17.87	5.51 ± 0.88	4.13 ± 0.31	4.86 ± 0.46
Prednisolone	0.13	2.50		15.17 ± 3.01	6.36 ± 1.52		7.71 ± 0.90		6.73 ± 0.24
Cortisol	0.25	0.75	7.48 ± 5.62	0.89 ± 0.98	1.18 ± 0.88	2.81 ± 5.62	3.08 ± 0.15	2.71 ± 0.02	2.72 ± 0.03
Tetrahydrocortisone	0.25	5.00	< MQL	< MQL	< MQL	< MQL	8.77 ± 0.67		
Prednisolone acetate	10.00	10.00	< MQL	< MQL	< MQL	< MQL			

were 1.5, 2.0 and 2.5 times the MQL. In absence of certified reference material, trueness was investigated by calculating the recovery. For all compounds, the recovery ranged between 97% and 109%, with RSDs below 10% (n = 70). These recoveries are better in comparison to literature, ranging in aquatic matrices from 88 to 120% [16,20,45].

The precision, covering the repeatability and within-laboratory reproducibility, was in line with the Horwitz equation. The RSDs of repeatability and within-laboratory reproducibility ranged, respectively, from 3.7 to 8.5% and 3.8–10.5% for all compounds (Table 2 and Table A.3.). These values are comparable to the RSDs that have been described in literature for a rather limited number of EDCs (Table 3), reporting repeatability RDSs from 4.2 to 8.3% and within-laboratory reproducibility RSDs from 3.6 to 12.0% [16,20,45].

3.2.5. Cross validation on freshwater

A cross-validation on fresh tap water samples was performed to extend the scope of the method and indicate its versatility (Table A.5.). Tap water is known for containing free chlorine, and is expected to have different matrix effects [46]. As compared to the performance characteristics obtained for seawater, similar results were achieved for fresh tap water. The robustness can be explained by the use of matrix-matched calibration curves and multiple suitable deuterated internal standards. Therefore, extending this multi-residue method to more complex aquatic matrices such as the influent and effluent of waste water treatment plants, is anticipated not to drastically alter its performance characteristics.

3.3. Application to seawater samples

The suitability of the developed method for target analysis of the 4 EDC subclasses, i.e. androgens, oestrogens, progestins and corticosteroids, was proven through the analysis of 28 seawater samples. The seawater samples were collected from four different locations at the BPNS, during two different sampling campaigns, each with four biological/physical replicates. The average concentrations of the four replicates are depicted in Table 4. The low standard deviations of the four biological/physical replicates confirm the fitness-for-purpose of the developed method for quantifying EDC residues in the marine environment. In addition, the quantified ranges of EDC-residues in the seawater samples confirm the need of applying MQL-levels during validation.

Regarding the multi-EDC profiling analysis, all the classes were ubiquitously present in the seawater samples. Besides the parent EDCs, different metabolites, transformation products, and or degradation products of testosterone, estradiol, and progesterone were quantified (i.e. dihydro, methyl, acetate, propionate, and benzoate form). The most abundant compounds quantified in the seawater samples were the corticosteroids.

4. Conclusions

A new analytical UHPLC-HR-Q-Orbitrap-MS multi-residue method was developed and successfully validated for the simultaneous quantification of 70 EDCs in sea and fresh water samples. The empirical MQLs in aquatic matrices for the androgens, oestrogens, progestins, and corticosteroids ranged respectively between 0.13 and 5.00 ng L^{-1}, 0.25–5.00 ng L^{-1}, 0.13–2.50 ng L^{-1}, and 0.50–5.00 ng L^{-1}. These low MQLs have shown to be necessary during the environmental application, due to the low concentration levels of EDCs residues.

The newly developed method may constitute an important tool for the holistic monitoring of the EDC contamination of aquatic environments. Moreover, the presented multi-residue method covers the most important EDC classes, and therefore fulfils the current lack of measuring progestins in environmental matrices. This will lead to a better understanding of the ecotoxicological implications of steroidal EDCs in the aquatic environment. Furthermore, the developed method offers the opportunity to screen a virtually unlimited number of (un)known compounds. Finally, monitoring a broad range of EDCs will contribute to the European Water Framework Directive, resulting in better regulations on environmental quality standard levels.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.07.001.

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