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Determination of 26 endocrine disrupting chemicals in fish and water using modified QuEChERS combined with solid-phase extraction and UHPLC-MS/MS†

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Endocrine-disrupting chemicals (EDCs) in the environment have adverse effects on humans and wildlife. A method based on ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) for the determination of 26 EDCs (including five estrogens, eight androgens, three progestogens, six glucocorticoids, two mineralocorticoids and two thyroid hormones) in fish and water was developed. Various experimental parameters that could affect the extraction efficiencies were investigated in detail. The sample was extracted by a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method with 20 mL of acetonitrile (for fish) or 5 mL of ethyl acetate (for water), and then cleaned-up using an Oasis HLB SPE (solid-phase extraction) cartridge. The analytes were quantified using an isotope-labelled internal standard and recoveries between 69.1% and 120.5% were obtained. The relative standard deviation of inter- and intra-day analyses for all the compounds was below 20%. The detection limits ranged from 0.01 to 0.98 ng mL^{-1} for water and 0.01 to 9.04 ng g^{-1} for fish. For real samples, progesterone and trenbolone were detected in zebrafish (*Danio rerio*) samples at 5.73 ± 0.21 and 7.45 ± 0.34 ng g^{-1}, respectively. There was no target analyte detected in tap-water samples. The developed method would be useful for monitoring EDC abuse in fishery, potential EDC screening and risk assessment in aquatic toxicology.

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1 Introduction

Endocrine-disrupting chemicals (EDCs) are typically identified as compounds that can interact with the endocrine system of organisms and thus act as agonists or antagonists of natural hormone action.[1] Hormones are one kind of the most important EDCs, which include natural and synthetic hormones. A wide range of EDCs, such as estrone (E1), 17β-estradiol (E2), testosterone (T), boldenone (BOL), and methyltestosterone (MT), have been found in surface water (lakes, rivers and drinking water) throughout the world including Asia,[2–4] Europe[5–7] and Oceania.[8] Incomplete removal of EDCs during waste water treatment was considered as an important issue.[9] Besides, synthetic hormones, such as diethylstilbestrol (DES), trenbolone (TB) and 19-nortestosterone (19-NT), are often illegally applied as growth promoters and repartitioning agents in meat-producing animals. These substances have been found in edible matrices, muscle, organ tissue, milk, *etc.*[10] Studies indicated that female mice treated neonatally with DES developed a high incidence of uterine adenocarcinoma,[11] and TB exposure caused rapid effects on plasma steroids and vitellogenin of fathead minnows, particularly in females.[12] So it is necessary to monitor EDC residues in the environment and different animals.

There are several methods reported in the literature for multi-residue detection of hormones by gas chromatography-mass spectrometry (GC-MS)[13–15] and liquid chromatography-tandem mass spectrometry (LC-MS/MS).[16–18] Generally, derivatization steps are frequently required in GC analysis to improve sensitivity by changing the chemical structure of analytes, which lead to higher ionisable molecules.[19] However, derivatization is time-consuming and complicated, which restricts the application of GC-MS to the simultaneous determination of several classes of steroids. Compared with GC-MS, LC-MS/MS is supposed to have high sensitivity and specificity without additional derivatization. In the past decade, some LC-MS/MS methods were developed to determine the residues of hormones in pork,[16,18] beef,[10,16,20] milk[19,21] and water.[20,22] But few

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of the developed multi-residue methods were focused on the detection of hormones in aquatic organisms.[23] Nowadays, model aquatic organisms (*e.g.* zebrafish (*Danio rerio*)) have been widely used for the ecotoxicological risk assessment of EDCs.[24] Previous studies revealed that exposure to some potential EDCs could influence the endocrine disruption system of zebrafish through the mRNA expression of genes.[25,26] However, there is no direct evidence from the content change of EDCs in zebrafish. It would be useful to develop a simple, fast and efficient method for EDC determination in fish, which would be helpful for EDC screening and risk assessment. Meanwhile, the developed method would be practical for monitoring EDCs in fisheries, in which some EDCs were abused to improve fish growth.

The general procedures of sample preparation in EDC determination previously involved solid/liquid extraction followed by cleaning-up with solid-phase extraction (SPE) and required the use of large amounts of organic solvents for each extraction and time for the preparation (30–60 min) of each sample.[10,27] A bottle neck in the trace analysis of EDCs in complex environmental samples (*e.g.* surface and waste water) is the absence of a sufficiently sensitive analytical procedure. Recently, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method has been widely applied to analyze pesticides in a variety of sample matrices, such as vegetables and fruits[28] and other foods.[29] The advantages of the QuEChERS method are that it is simple, rapid and requires low solvent consumption, which allow it to determine hormones in wastewater,[30] soil[31] and other matrices.[15,32] However, the procedure is relatively new for the fish matrix, and there are few studies published previously based on the QuEChERS method for detection of EDCs.[33] Most of the currently available analytical methods usually can simultaneously analyze dozens of EDCs, all of which belong to the same class or a few classes.[16,18,20] Moreover, in previously developed methods, most of the target compounds were limited to a few certain hormones illegally added,[34] whereas it is little known about detection of natural hormones with LC-MS/MS due to matrix complexity and low background levels (ng kg^{-1} to mg kg^{-1}). It would be useful to monitor not only natural hormones and their metabolites, but also artificial hormones simultaneously, since natural metabolic patterns may be disrupted by these EDCs. The development of a highly sensitive analytical method which can simultaneously determine various kinds of EDCs (estrogens, androgens, progestogens, glucocorticoids, mineralocorticoids and thyroid hormones) in water and fish by LC-MS/MS is the goal of this study.

2 Experimental

2.1 Reagents and chemicals

Cortisone, dehydroepiandrosterone (DHEA), 21-hydroxyprogesterone (21-OHP), MT, betamethasone (B), DES, E1, estriol (EST), ethynylestradiol (EE2), aldosterone (A), 17-hydroxypregnenolone (Δ5-17-OHP), estriol-d_3 (EST-d_3) and stanozolol-d_3 (ST-d_3) were purchased from J&K Scientific (Shanghai, China); 17-hydroxyprogesterone (17-OHP) and cortexolone from TCI (Shanghai, China); 3,3,5-triiodo-L-thyroxine (T3), L-thyroxine (T4), E2, T, corticosterone, 19-NT, BOL, androstenedione (AN), progesterone (P4), hydrocortisone (Hd) and dexamethasone (Dex) from Aladdin (Shanghai, China); and stanozolol (ST), TB, estradiol-d_3 (E2-d_3), and progesterone-d_9 (P4-d_9) from Sigma-Aldrich (St. Louis, USA). Analytical standards of ≥98% purity were used. The chemical structures of target analytes are shown in Fig. S1 (ESI†).

Acetonitrile and methanol of HPLC grade were obtained from Merck (Darmstadt, Germany); formic acid from Tedia (Fairfield, USA); ammonia (25%, w/v), hexane, ethyl acetate, anhydrous magnesium sulfate, anhydrous sodium sulphate, sodium chloride, and aluminium oxide from Sinopharm (Shanghai, China); and primary/secondary amine (PSA) from Welchrom (Shanghai, China). Ultrapure water was generated using a water purification system (Pall Corporation, USA). Dialysis tubing (Spectra/Por 6) of regenerated cellulose with a molecular exclusion size of 1000 Da was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA); Bond Elut-FL (500 mg, 3 mL) cartridges from Agilent Technologies (Bellefonte, PA, USA); Oasis HLB (60 mg, 3 mL) SPE cartridges from Waters Company (Milford, MA, USA); and C18 (500 mg, 6 mL) SPE cartridges from Sipore Company (Dalian, China).

ASE-12 solid-phase extraction and nitrogen evaporators MTN-5800 were obtained from Auto Science Company (Tianjin, China). Centrifuge Anke DL-5-B (Shanghai flying pigeon company, China) and Vortex WH-861 (Hualida, China) were used for extraction.

2.2 Preparation of standard solutions

The standard stock solutions at a concentration of 100 mg L^{-1} were prepared for the 26 target compounds and four isotope-labelled internal standards (ISs) in methanol. Mixed standard working solutions in the concentration range of 1.0 to 400 ng mL^{-1} were prepared by mixing and diluting each stock solution with methanol for plotting calibration curves. Correspondingly, quality control standards (QCs) were obtained by adding 10 μL of the corresponding spiking mixed standard solutions to 990 μL of blank sample extracts (zebrafish and tap-water). In addition, the mixtures of ISs at a concentration of 0.5 mg L^{-1} were made by mixing and diluting each IS stock solution with methanol and applied as 0.1 mL to all samples prior to extraction. For quality assurance, considering the fact that the existence of endogenous hormones (*e.g.* P4) in the fish samples could not be completely excluded, and thus fish blank samples with no target analytes or low levels of target analytes and water blank samples from the tap-water in the laboratory were extracted with each batch of fortified samples. All solutions and matrices were stored at −20 °C until analysis.

2.3 Sample preparation

Water samples were from tap-water in the laboratory. Adult zebrafish were obtained from a local fish market (Hangzhou, China) and they were stored at −20 °C until analysis. Fish samples were homogenized (*ca.* 100 g) immediately before analysis. All procedures were conducted in accordance with the guidelines for the care and use of laboratory animals of the

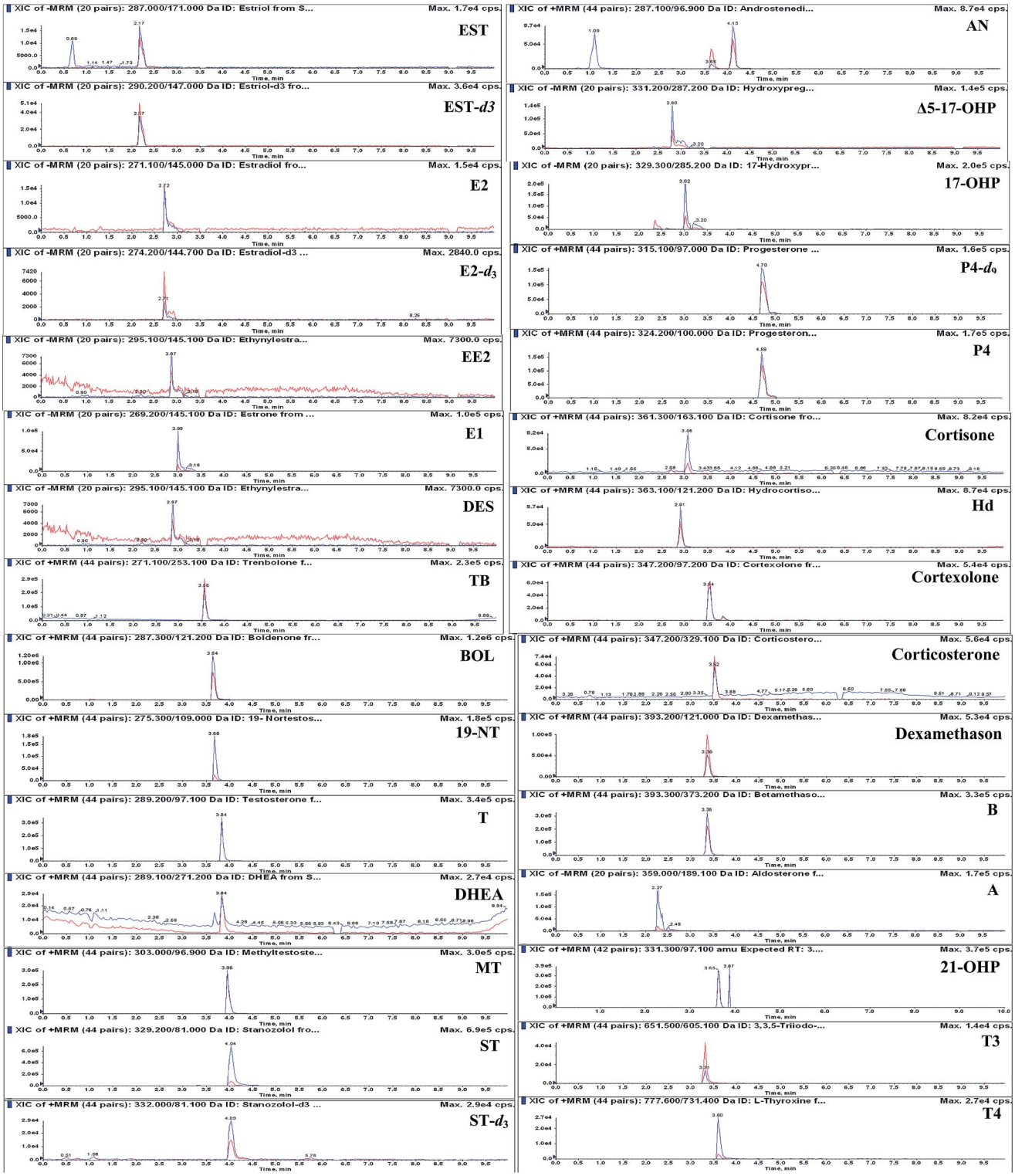

Fig. 1 The MRM chromatograms of each target compound in standard solution at 50 ng mL^{-1}.

National Institute for Food and Drug Control of China. The streamlined procedure given below was used for extraction and clean-up in the final method.

2.3.1 QuEChERS extraction. Five grams of a homogenized fish sample or 5 mL of tap-water were transferred into a 50 mL polypropylene centrifuge tube and spiked with 100 μL internal standards (0.5 mg L^{-1}). In addition, the samples for the recovery test were spiked with a certain amount of mixed standard solution. For fish samples, 4 g of anhydrous Na_2SO_4, 1 g of NaCl, 2 mL of water and 20 mL of acetonitrile were added to the tube.

Table 1 MRM conditions for the target compounds: retention time (R_t), precursor ion (Q1), product ions (Q3), DP (declustering potential), EP (entrance potential), CE (collision energy), and CXP (collision cell exit potential)

Category	Compound	Abbreviations	Internal standards	Ion source	R_t (min)	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Estrogens	Estriol	EST	EST-d_3	ESI−	2.3	287	171[a]	−9.1	−8.7	−45	−19
							144.9			−58	−16
	Estriol-d_3	EST-d_3	—	ESI−	2.3	290.2	147	−25	−9	−55	−17
							173.1[a]			−49	−9
	Estradiol	E2	E2-d_3	ESI−	3.3	271.1	145[a]	−21.6	−11.9	−61.5	−7.9
							183			−61.6	−18.9
	Estradiol-d_3	E2-d_3	—	ESI−	3.2	274.2	144.7	−65	−2	−65	−8.8
							185.1[a]			−48.8	−21.5
	Ethynylestradiol	EE2	E2-d_3	ESI−	3.5	295.1	145.1[a]	−19.2	−11.7	−60	−8.1
							158.9			−55.2	−9.2
	Estrone	E1	E2-d_3	ESI−	3.7	269.2	145.1[a]	−88	−7	−61	−17
							159			−49.9	−23.1
	Diethylstilbestrol	DES	E2-d_3	ESI−	3.8	267	251	−20.3	−9.3	−36	−29
							237.1[a]			−41.2	−7.1
Androgens	Trenbolone	TB	ST-d_3	ESI+	3.0	271.1	253.1	48	9	21	31
							199.2[a]			29	17
	Boldenone	BOL	ST-d_3	ESI+	3.1	287.3	121.2[a]	79.7	3	44.8	6.2
							135.1			24.5	8.9
	19-Nortestosterone	19-NT	ST-d_3	ESI+	3.3	275.3	109[a]	8.8	3.9	38.7	12.3
							145			41	37
	Testosterone	T	ST-d_3	ESI+	3.5	289.2	97.1	15.9	9.9	35.3	15.9
							109[a]			36.7	14
	Dehydroepiandrosterone	DHEA	ST-d_3	ESI+	3.5	289.1	271.2	36	12.2	17	25
							253.2[a]			20.8	30
	Methyltestosterone	MT	ST-d_3	ESI+	3.8	303	96.9[a]	36.6	3	39.4	15.2
							109.1			41.2	10.1
	Stanozolol	ST	ST-d_3	ESI+	3.9	329.2	81[a]	8.4	4	79.2	14
							121			66.5	12
	Stanozolol-d_3	ST-d_3	—	ESI+	3.9	332	81.1[a]	15	6	50	10
							95			53	14
	Androstenedione	AN	ST-d_3	ESI+	3.9	287.1	96.9[a]	70.2	8.6	32.1	19.1
							109			41.1	16.1
Progesterones	17-Hydroxypregnenolone	Δ5-17-OHP	P4-d_9	ESI−	3.6	331.2	287.2[a]	−15	−4	−29	−9
							313.1			−29	−45
	17-Hydroxyprogesterone	17-OHP	P4-d_9	ESI−	3.9	329.3	285.2[a]	−16	−3	−34	−17
							301.3			−31	−18
	Progesterone-d_9	P4-d_9	—	ESI+	5.5	324.2	100[a]	9	3	27	18
							113.1			36	6
	Progesterone	P4	P4-d_9	ESI+	5.6	315.1	97	75.6	3.7	29	11.5
							109.1[a]			40.3	6.9
Glucocorticoids	Cortisone	—	ST-d_3	ESI+	2.1	361.3	163.1[a]	36	5	35	10
							105			53	13
	Hydrocortisone	Hd	ST-d_3	ESI+	2.5	363.1	121.2[a]	3.3	6.5	35.5	12.8
							327.2			21	19.7
	Cortexolone	—	ST-d_3	ESI+	2.6	347.2	97.2	20	6	38	6

Table 1 (*Contd.*)

Category	Compound	Abbreviations	Internal standards	Ion source	R_t (min)	Q1 (*m*/*z*)	Q3 (*m*/*z*)	DP (V)	EP (V)	CE (V)	CXP (V)
							109[a]			50	10
	Corticosterone	—	ST-d_3	ESI+	2.6	347.2	329.1[a]	21	7	29	21
							121.1			35	12
	Dexamethasone	Dex	ST-d_3	ESI+	2.8	393.2	121	30.2	7.1	69.3	16.1
							147.2[a]			46.6	6.9
	Betamethasone	B	ST-d_3	ESI+	2.8	393.3	373.2[a]	37	6.5	16.8	23
							355.2			20	26
Mineralocorticoids	Aldosterone	A	EST-d_3	ESI−	2.4	359	189.1[a]	−63.4	−2.8	−35	−10.2
							174			−59.6	−5
	21-Hydroxyprogesterone	21-OHP	P4-d_9	ESI+	3.3	331.3	97.1	68.3	9	37.5	9.2
							109[a]			47	7
Thyroid hormones	3,3,5-Triiodo-L-thyroxine	T3	P4-d_9	ESI+	2.4	651.5	605.1	14.4	12	30	17
							478.8[a]			53.8	25.2
	L-Thyroxine	T4	P4-d_9	ESI+	2.8	777.6	731.4[a]	16.1	10.1	39.6	21
							604.9			58.1	8

[a] MRM transition used for quantification.

For tap-water samples, 5 mL of ethyl acetate were added to the tube. The supernatant was collected after being vortexed for 1 min and then centrifuged at 4000 rpm for 10 min at 4 °C. And the organic layer was transferred into a pear-shaped evaporation flask carefully. Subsequently, the above extraction procedure was repeated one time from the addition of the acetonitrile (fish samples) or ethyl acetate (water samples) step. The resulting supernatant was merged and evaporated using a water bath at 40 °C. The residue was dissolved in 5 mL of methanol : water (5 : 95, v/v) for subsequent SPE clean-up.

2.3.2 SPE optimization protocol. To ensure maximum recovery of target analytes, SPE parameters including cartridges, elution solvent and ionic strength were optimized. To study the retention capacity of target compounds on various sorbents, break-through recoveries were tested as follows: prior to sample loading, C18 and HLB cartridges were preconditioned with 4 mL of methanol and 4 mL of water successively. Florisil cartridges were conditioned with 5 mL of *n*-hexane and *n*-hexane : acetone (90/10, v/v), successively. The targeted fractions were collected after the mixed standard solution (1 mg L^{-1}, diluted with 5 mL of methanol : water (5 : 95, v/v)) was loaded onto these cartridges. And the targeted fractions were dried under a stream of nitrogen at 40 °C. The dried residues were reconstituted in 1 mL of acetonitrile. Next, the organic solvent strength and volume of the eluent were optimized for HLB by using 2 mL per time of mixture solution (methanol/water) with an increment content of methanol from 10% to 100% (all v/v). Each 2 mL of the targeted fractions were collected and analysed.

2.3.3 SPE final protocol. An HLB cartridge was conditioned sequentially with 4 mL of methanol and 4 mL of water. After 5 mL of sample solution was loaded, the cartridge was washed with 5 mL of 30% methanol/water solution (30 : 70, v/v) and dried with a vacuum pump. The crude analytes were eluted with 6 mL of methanol : water : ammonium (80 : 16 : 4, v/v/v). The eluate was dried under a gentle nitrogen stream at 40 °C. The residue was dissolved with 1 mL of acetonitrile and filtered using a 0.2 μm one-off PTFE syringe filter prior to UHPLC-MS/MS analysis.

2.4 UHPLC-MS/MS analysis

The UHPLC-MS/MS was composed of a 5500 QTRAP MS/MS system (AB SCIEX, Singapore) and an Eksigent ekspert ultra LC 100-XL system (AB SCIEX, the Netherlands). Data were processed with Analyst 1.6.1 software. For LC analysis, a LC 100-XL system with a binary pump and an autosampler was employed. All analytes were separated using an Endeavorsil C18 column (100 mm × 2.1 mm, 1.8 μm pore size, Dikma, USA). The temperature of the column oven was held at 40 °C and the injection volume was 5 μL. Water (A) and purified acetonitrile (B) were used as mobile phases at a flow rate of 0.3 mL min^{-1}. The binary gradient was programmed as follows: 0 min, 30% B; 1.5 min, 55% B; 3 min, 63% B, constant for 3 min; 8 min, 85% B, constant for 1 min; and 10 min, 30% B. The 5500 QTRAP MS/MS system was equipped with an electrospray ionization (ESI) source. Nitrogen was used as the nebulizer and collision gas. Unit mass resolution was set in both mass-resolving

quadrupoles Q1 and Q3. The ESI source in positive mode was as follows: ion spray (IS) voltage: 5500 V; nitrogen collision gas (CAD): 8 psi; curtain gas: 35 psi; nebulizer gas (GS1): 40 psi; auxiliary gas (GS2): 50 psi; source temperature: 550 °C. The ESI source in negative mode was as follows: IS voltage: −4500 V; CAD: 8 psi; curtain gas: 40 psi; GS1: 50 psi; GS2: 50 psi; source temperature: 550 °C. The separation of each target analyte under the optimized conditions was determined within 10 min (Fig. 1). Optimization of targets was performed by manual tuning, namely injecting individual standard solutions directly into the source. A multiple reaction monitoring (MRM) transition optimised with the protonated/deprotonated molecular ion selected as the precursor, and the most abundant product ion was used for quantification. A second transition was selected for all compounds for confirmatory purposes. The optimized MS parameters including declustering potential (DP) and entrance potential (EP) for precursor ions, collision energy (CE) and collision cell exit potential (CXP) for product ions are summarized in Table 1.

3 Results and discussion

3.1 QuEChERS modification

Many studies indicated that enzymatic hydrolysis in the sample preparation procedure did not improve the recovery of free hormones in muscle tissue.[35–37] Therefore, enzymatic hydrolysis was not used in this study. The selection of an appropriate extraction solvent is of importance for the QuEChERS extraction. Some common organic solvents, such as *n*-hexane, ethyl acetate, methanol and acetonitrile, were tested for investigating the extraction efficiency. Acetonitrile and ethyl acetate provided better extraction efficiency for all analytes with recoveries in the range of 80–102%. Compared with ethyl acetate, acetonitrile precipitation is a better way to remove proteins from animal samples. Hence, acetonitrile was selected as the extraction solvent for fish samples, while ethyl acetate was for water samples. Different amounts of $MgSO_4$ and NaCl were tested and, in our case, the results showed negligible differences in terms of recovery factors but an improvement in terms of interfering peaks when 4 g $MgSO_4$ and 1 g NaCl were used. Extraction volume is another important factor to obtain efficient extraction. It was found that when 20 mL of acetonitrile and 5 mL of ethyl acetate were used for fish and water samples, respectively, acceptable recoveries of the analytes were produced. In the QuEChERS method, the purification step was generally performed by dispersive SPE (dSPE). However, it was found that PSA and alumina sorbents were not efficient enough for the reduction of matrix effects in our study, which was consistent with the result of ref. 31. It was therefore necessary to use a clean-up step with SPE cartridges.

3.2 Selection of SPE cartridges

After the extraction, the purification step plays a vital role in the analysis of EDCs because of their low concentration in the tested sample and complex matrices. In the case of analyzing various kinds of hormones, more than one SPE cartridge was usually needed for the enrichment and separation of compounds.[10,18] The use of various SPE cartridges makes the sample preparation process tedious and costly. Furthermore, multiple steps in the sample preparation may increase the loss of the compounds in tested samples and reduce the recovery and analysis accuracy, especially on trace residues analysis. There were few studies which can simultaneously clean up more than six classes of EDCs simultaneously with a single SPE cartridge. In this work, different SPE cartridges were compared to select the optimal one to develop a simple multi-method for various classes of EDCs.

Based upon pK_a and log *P* of targeted compounds (referenced by DrugBank[38]), Florisil, C18 and HLB were used to select a suitable SPE cartridge for removing matrix components. Florisil is considered as a normal-phase polar sorbent, while C18 and HLB belong to reversed-phase cartridges. SPE breakthrough of standard solution load was investigated prior to validation to evaluate the retention capacity of target compounds on SPE cartridges. The result in Fig. 2 showed that progesterones, glucocorticoids and thyroid hormones had a better retention capacity for all three cartridges. Higher breakthrough recoveries were observed from estrogens and mineralocorticoids, especially EE2 (20.8%), using the Florisil SPE cartridge. Compared with C18, HLB had a slightly better retention capacity for most analytes. In addition, HLB, with its hydrophilic–lipophilic balance, is versatile and efficient for the extraction of EDCs with a wide range of polarities and pH

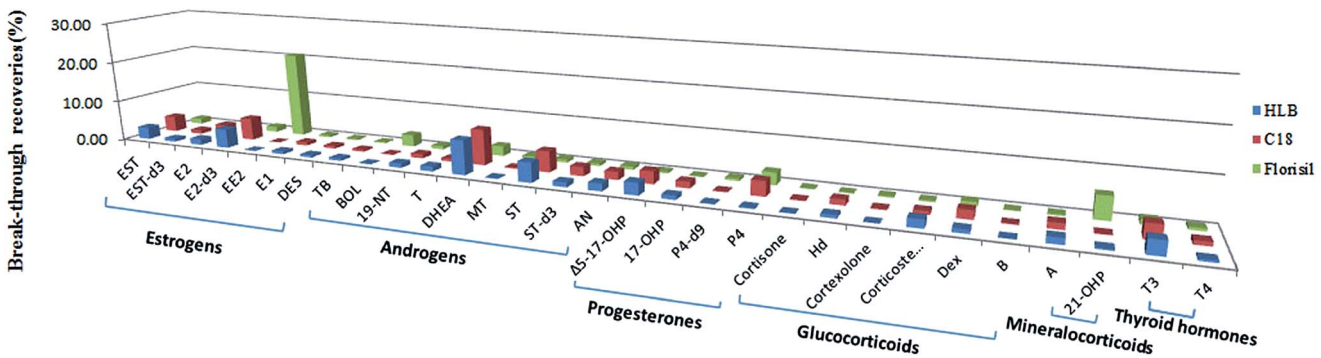

Fig. 2 Break-through recoveries of analytes using various SPE cartridges (1 mg L^{-1}, $n = 2$).

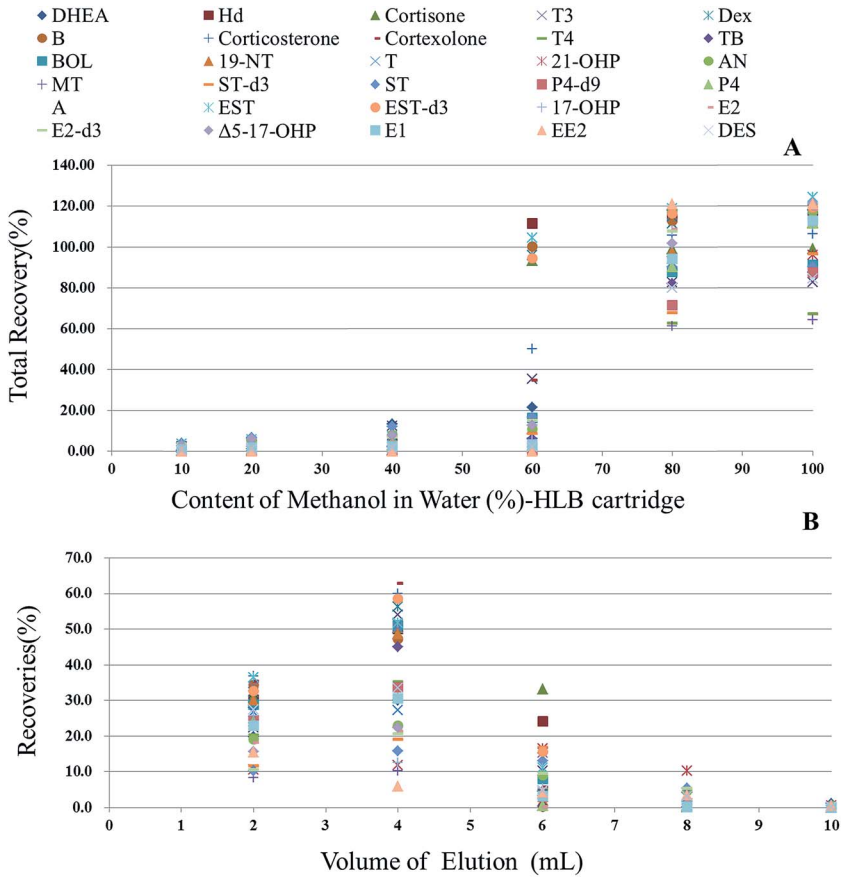

Fig. 3 Optimization of SPE eluting solvent. A: elution profile of analytes for HLB cartridges (1 mg L^{-1}, $n = 2$); B: elution curve of analytes for HLB cartridge (1 mg L^{-1}, $n = 2$).

values. HLB has been used in many studies with different kinds of water samples.[20,39] Yang *et al.*[10] detected 50 hormones in muscles (pork, beef and shrimp) and purified them using graphitized carbon-black and NH_2 SPE cartridges, when the average recoveries were 76.9–121.3% and the relative standard deviation was 2.4–21.2%. However, HLB cartridges showed the high retention capacity of ECDs and effective removal of proteins and polar lipids in fish in our study, which might have

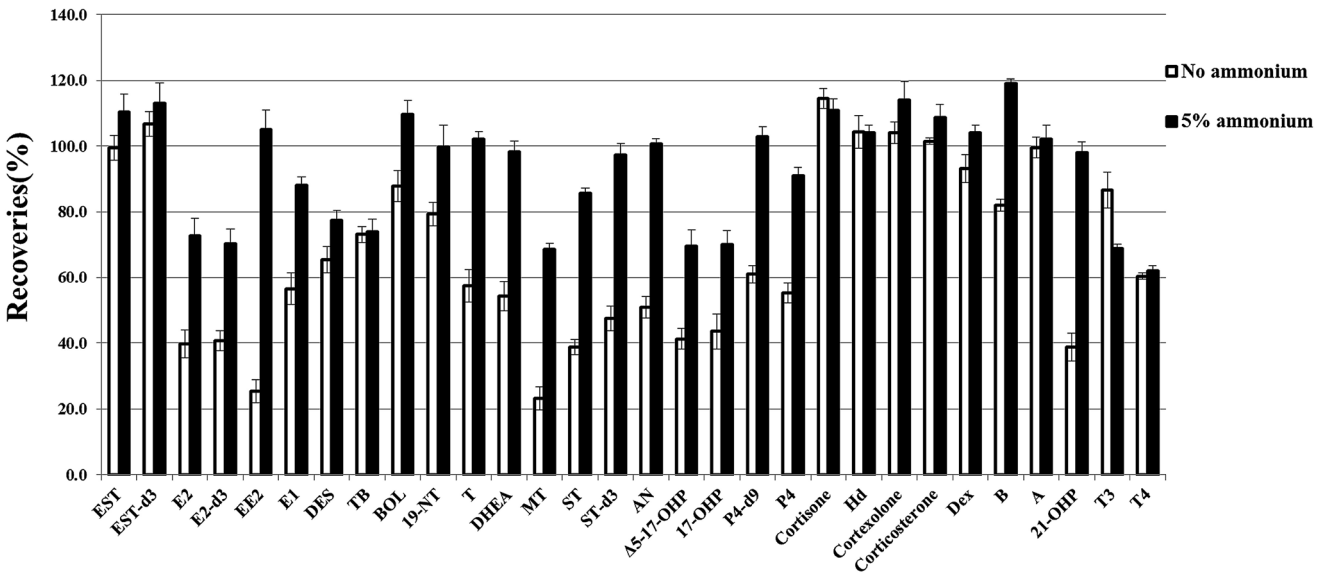

Fig. 4 The effect of elution ionic strength on the HLB SPE cartridge (1 mg L^{-1}, $n = 2$).

Table 2 The recoveries and precision of the LC/MS/MS method ($n = 5$) and RSD for inter-day precision

	Zebrafish			Tap-water		
Compound	Spiked (ng g^{-1})	Recovery (%)	RSD (%)	Spiked (ng mL^{-1})	Recovery (%)	RSD (%)
EST	10.0	90.3	10.3	1.0	90.4	5.4
	50.0	84.6	6.2	5.0	86.6	13.8
	100.0	88.3	6.3	10.0	77.6	7.8
EST-d_3	1.0	79.6	12.5	1.0	116.7	3.7
	5.0	89.3	10.4	5.0	70.6	5.3
	10.0	80.5	9.4	10.0	77.2	4.9
E2	5.0	118.6	12.4	1.0	82.7	8.2
	10.0	106.6	13.2	5.0	100.0	13.8
	50.0	80.4	10.0	10.0	86.6	10.1
E2-d_3	10.0	98.5	11.2	1.0	106.9	14.4
	50.0	115.8	9.5	5.0	90.2	5.3
	100.0	108.3	8.0	10.0	80.3	7.9
EE2	2.0	99.2	10.3	1.0	117.1	3.2
	5.0	86.3	11.2	5.0	73.9	6.5
	10.0	82.0	7.5	10.0	96.9	4.9
E1	1.0	104.3	12.2	1.0	98.8	11.6
	5.0	79.4	11.0	5.0	90.1	2.4
	10.0	73.9	7.9	10.0	97.0	8.4
DES	1.0	95.8	5.2	1.0	90.3	14.3
	5.0	87.5	6.7	5.0	116.8	6.0
	10.0	92.0	4.5	10.0	91.6	4.9
TB	1.0	97.4	9.4	1.0	81.6	10.1
	5.0	105.0	9.9	5.0	81.8	6.1
	10.0	84.9	3.3	10.0	78.9	8.0
BOL	1.0	114.9	6.4	1.0	82.1	10.2
	5.0	108.9	4.0	5.0	74.6	0.2
	10.0	104.0	5.7	10.0	78.2	8.2
19-NT	1.0	76.6	9.8	1.0	93.4	12.5
	5.0	105.0	9.9	5.0	96.1	10.2
	10.0	72.5	7.9	10.0	81.6	7.1
T	1.0	118.8	8.4	1.0	99.4	1.4
	5.0	86.9	10.4	5.0	82.8	6.4
	10.0	86.3	3.4	10.0	84.9	13.4
DHEA	2.0	108.6	5.5	1.0	98.4	10.1
	5.0	81.3	3.9	5.0	95.8	12.3
	10.0	114.6	7.1	10.0	83.5	0.3
MT	1.0	98.3	8.1	1.0	115.1	15.0
	5.0	84.8	4.5	5.0	98.0	4.6
	10.0	81.2	2.0	10.0	77.9	5.5
ST	1.0	102.3	13.0	1.0	111.9	15.0
	5.0	85.3	10.5	5.0	91.4	4.7
	10.0	80.1	4.6	10.0	84.0	8.9
ST-d_3	1.0	117.5	9.7	1.0	104.5	8.4
	5.0	83.8	10.6	5.0	90.2	8.9
	10.0	81.4	7.2	10.0	78.8	10.5
AN	1.0	89.9	10.7	1.0	117.1	10.4
	5.0	76.7	6.1	5.0	96.7	1.8
	10.0	73.0	3.9	10.0	97.0	4.3
Δ5-17-OHP	1.0	105.2	6.8	1.0	72.6	14.5
	5.0	101.4	11.7	5.0	88.7	6.4
	10.0	87.1	5.2	10.0	78.3	9.7
17-OHP	1.0	107.6	13.3	1.0	109.4	11.8
	5.0	95	14.8	5.0	96.8	6.9
	10.0	74.7	5.1	10.0	83.5	11.0
P4-d_9	1.0	101.5	12.8	1.0	91.2	2.7
	5.0	100.8	7.3	5.0	106.2	7.4
	10.0	98.7	8.7	10.0	91.0	8.7
P4	1.0	102.3	13.5	1.0	85.5	5.9
	5.0	87.0	5.4	5.0	84.5	12.4
	10.0	104.0	10.8	10.0	82.7	12.0
Cortisone	1.0	114.1	14.2	1.0	70.3	7.2
	5.0	101.1	10.4	5.0	73.6	12.6

Table 2 (*Contd.*)

	Zebrafish			Tap-water		
Compound	Spiked (ng g^{-1})	Recovery (%)	RSD (%)	Spiked (ng mL^{-1})	Recovery (%)	RSD (%)
	10.0	101.1	5.9	10.0	70.7	8.2
Hd	1.0	117.8	8.9	1.0	98.3	7.9
	5.0	86.9	3.6	5.0	80.8	14.9
	10.0	93.2	2.6	10.0	85.9	10.0
Cortexolone	1.0	100.2	10.3	1.0	97.0	1.5
	5.0	80.3	11.8	5.0	71.1	5.1
	10.0	79.4	7.6	10.0	72.0	3.4
Corticosterone	1.0	104.3	10.2	1.0	94.1	13.2
	5.0	89.6	6.8	5.0	88.3	12.7
	10.0	90.5	7.2	10.0	79.8	8.9
Dex	1.0	103.2	12.5	1.0	82.2	14.8
	5.0	90.9	9.1	5.0	70.4	12.3
	10.0	89.5	6.3	10.0	73.7	5.3
B	1.0	110.5	8.2	1.0	104.6	7.2
	5.0	76.8	6.0	5.0	87.1	6.5
	10.0	72.6	5.3	10.0	89.3	4.8
A	1.0	100.0	13.6	1.0	101.4	10.3
	5.0	89.7	8.4	5.0	89.1	12.7
	10.0	84.7	6.8	10.0	81.4	8.0
21-OHP	1.0	86.0	9.2	1.0	91.8	10.2
	5.0	86.9	6.7	5.0	73.4	4.8
	10.0	110.3	10.0	10.0	69.6	6.4
T3	1.0	103.4	8.3	1.0	98.1	10.3
	5.0	98.2	10.4	5.0	99.6	5.4
	10.0	89.5	5.6	10.0	89.4	6.9
T4	1.0	96.3	6.2	1.0	102.5	8.5
	5.0	95.2	7.1	5.0	89.3	6.1
	10.0	80.8	5.4	10.0	95.7	7.4

a better performance, instead of more than one SPE cartridge. Taking account of expensive SPE cartridges, one single HLB was chosen for further optimization.

3.3 Optimization of eluting solvent

In the extraction step, desorption is greatly influenced by the solvent type used. For the HLB SPE cartridge, the solvent must have enough strength for stripping off the target compounds from the sorbent phase completely, as well as, minimizing polar interference from complex matrices. As shown in Fig. 3A, most analytes were retained on the HLB cartridge when methanol was less than 40%, and then they were gradually eluted with increasing proportions of methanol in the eluent. After percentages of methanol in water reached 80% and 100% (v/v), satisfactory total recoveries of the 26 analytes using HLB cartridges can be obtained (61.08–120.89% and 64.17–121.21%, respectively), while purification effects were better for 80% methanol, which were then selected for the following SPE procedure.

The analytes (DHEA, T3, AN, ST, A, EST, E2-d_3 and Δ5-17-OHP) exhibited recoveries of 5–12% when they were eluted with 40% methanol, which indicated that they would have lower recoveries and retain on HLB cartridges through reducing the percentage of methanol in elution solvents. Furthermore, we found that the recoveries of some analytes with 30% methanol in elution solvents were slightly higher than those with 20% methanol, but obviously lower than those with 40% methanol. Thus, prior to elution with 80% methanol, a washing step of 30% (v/v) methanol/water (4 mL) was set to remove interferents. The optimization of elution solvent volumes was the next step for HLB cartridges, and the results in Fig. 3B showed that the recoveries of 26 hormones in standard solution were about 10% and 1% when the volumes of the mixture solvent were 8 and 10 mL, respectively. Then the results (the data were not shown) for optimization of the volumes of elution solvents in each sample matrix also showed that elution with 6 mL of 80% methanol produced better recoveries of analytes. When the volume of the solvent was more than 6 mL, it would result in more interferents. Thus 6 mL mixture solvent was enough to elute the analytes from the SPE cartridge.

3.4 Optimization of pH

Previously, the washing procedures, including an organic wash and adjustment of pH, were efficient in reducing or eliminating matrix interference.[40] Thus, the next study was focused on the pH effect on SPE efficiency for EDCs, where ammonium was

Table 3 Validation of the analytical method for each target compound in the corresponding matrices: matrix effect (ME), linear dynamic range (LDR), coefficient of determination (r^2), limit of detection (LOD), and limit of quantification (LOQ)

	Zebrafish					Tap-water				
Compound	ME (%)	LDR (ng mL^{-1})	r^2	LOD (ng g^{-1})	LOQ (ng g^{-1})	ME (%)	LDR (ng mL^{-1})	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
EST	82.6	40–400	0.9987	9.04	30.12	82.3	1.0–100	0.9989	0.31	1.02
EST-d_3	119.1	5.0–100	0.9953	0.86	2.88	82.0	1.0–100	0.9901	0.26	0.88
E2	80.3	20–200	0.9993	5.17	17.24	119.7	5.0–100	0.9906	0.98	3.27
E2-d_3	107.2	30–300	0.9917	7.69	25.64	104.2	5.0–100	0.9925	0.91	3.03
EE2	120.6	10–100	0.9999	2.26	7.54	103.1	5.0–100	0.9966	0.87	2.56
E1	81.5	5.0–100	0.9996	0.96	3.19	119.7	1.0–100	0.9992	0.24	0.79
DES	82.5	5.0–100	0.9968	0.65	2.17	116.0	1.0–100	0.9996	0.05	0.17
TB	114.8	1.0–100	0.9964	0.30	0.99	96.0	1.0–100	0.9996	0.04	0.15
BOL	103.2	1.0–100	0.9968	0.12	0.41	119.4	1.0–100	0.9960	0.01	0.02
19-NT	103.3	5.0–100	0.9974	0.73	2.42	96.1	1.0–100	0.9910	0.03	0.09
T	86.0	1.0–100	0.9990	0.31	1.02	96.4	1.0–100	0.9957	0.01	0.04
DHEA	110.9	10.0–100	0.9981	3.01	10.04	82.5	1.0–100	0.9984	0.31	1.02
MT	90.7	5.0–100	0.9916	0.51	1.72	86.8	1.0–100	0.9934	0.07	0.22
ST	117.4	1.0–100	0.9967	0.07	0.25	88.2	1.0–100	0.9966	0.01	0.04
ST-d_3	87.3	1.0–100	0.9980	<0.01	0.01	87.8	1.0–100	0.9984	0.09	0.29
AN	77.9	1.0–100	0.9984	0.30	1.00	105.3	1.0–100	0.9940	0.07	0.23
Δ5-17-OHP	81.2	1.0–100	0.9987	0.09	0.30	104.1	1.0–100	0.9938	0.15	0.49
17-OHP	113.2	1.0–100	0.9985	0.14	0.46	117.5	1.0–100	0.9935	0.09	0.29
P4-d_9	87.9	5.0–100	0.9945	0.61	2.03	100.6	1.0–100	0.9968	0.02	0.07
P4	74.4	1.0–100	0.9909	0.32	1.07	113.8	1.0–100	0.9956	0.02	0.08
Cortisone	118.0	1.0–100	0.9922	0.09	0.30	115.9	1.0–100	0.9960	0.13	0.42
Hd	119.7	1.0–100	0.9953	0.21	0.69	113.4	1.0–100	0.9976	0.20	0.66
Cortexolone	115.8	1.0–100	0.9994	0.03	0.10	100.7	1.0–100	0.9960	0.03	0.09
Corticosterone	112.1	1.0–100	0.9985	0.10	0.34	90.7	1.0–100	0.9972	0.01	0.05
Dex	118.7	5.0–100	0.9988	0.50	1.68	103.5	1.0–100	0.9917	0.03	0.09
B	111.2	1.0–100	0.9999	0.26	0.86	103.7	1.0–100	0.9925	<0.01	0.01
A	73.6	5.0–100	0.9980	0.57	1.89	80.3	1.0–100	0.9967	0.28	0.93
21-OHP	120.2	1.0–100	0.9986	0.07	0.22	84.8	1.0–100	0.9922	0.02	0.06
T3	114.1	1.0–100	0.9926	0.14	0.46	96.5	1.0–100	0.9998	0.12	0.39
T4	119.7	5.0–100	0.9985	0.41	1.38	86.6	1.0–100	0.9969	0.14	0.45

tested. This parameter has a great influence on recovery yields since sample pH influences ionic strength and the affinity of target analytes to the sorbent phase. Fig. 4 shows that most analytes reached much better recoveries at 5% ammonium addition compared with no ammonium in the elution. For the estrogens and progestogens, the recovery of each analyte increased in the case of 5% ammonium, especially EE2. The recoveries of androgens (except TB, BOL, and 19-NT) obviously increased from about 50% to about 100% after 5% ammonium was applied into the elution, whereas some slight changes were observed on glucocorticoids and thyroid hormones. The various structures of EDCs caused the differences on the recoveries in the presence of a low proportion of ammonium. The alkaline conditions were favorable for the ionization of EDCs, thus reducing their affinity for the HLB sorbent and facilitating elution. Meanwhile, ammonium addition resulted in signal enhancement, which was further supported by suppression and enhancement effect change with pH adjustment in the elution step using ammonium hydroxide.[41] Therefore, a low proportion of ammonium could reduce matrix interference, consistent with the results of Gineys *et al.*[40] for improving the purification effect on soil with ammonia in the elution step. The data obtained demonstrated that the pH control is essential in order to enhance the migration of the EDCs to the sorbent phase.

3.5 Method validation

The method was validated using internal calibrations following peak areas of target analytes and internal standards (A/A_{IS}) against relative concentrations of target and internal standard compounds (C/C_{IS}). Calibration curves were constructed for most target analytes from 1.0 to 100 ng mL^{-1} (standard concentration levels at 1.0, 5.0, 10, 50 and 100 ng mL^{-1}) and the correlation of $r^2 > 0.99$ for all validation batches was obtained over these ranges. QCs ($n = 5$ of QC_1, QC_2 and QC_3) were prepared to evaluate intra- and inter-day levels of precision, and to evaluate the efficiency of analyte recoveries at low (QC_1), middle (QC_2) and high concentrations (QC_3) (Table 2). Percentage recoveries for fish ranged from 72.5 to 118.8 and for water from 70.3 to 117.1. The intra-day repeatability and inter-day reproducibility were expressed as relative standard deviation (RSD, %) for each concentration. The inter-day analyses were performed for the same three concentrations on three days, in the RSD range of 0.3–15.0% (Table 2). All of the RSD% values for intra-day were below 20% (the data are not shown).

Table 4 The methods for determination of EDCs with references

The types of EDCs	The amount of EDCs	Extraction process	Volume/weight	Matrix Sample	Recovery (%)	Detection method	References
Estrogens, androgens, progesterones, glucocorticoids, mineralocorticoids and thyroid hormones	26	Oasis HLB SPE	5 g/5 mL	Water Fish	70.3–117.1 72.5–118.8	UHPLC-MS/MS	Our present study
Estrogens, androgens, progesterones and other (propionate)	14	C18, Si and NH_2 SPE	5 g	Beef	66.4–115.2	LC-MS/MS	43
Androgens, progesterones and glucocorticoids	10	MSPD[a]	1 g	Chicken Pork Beef Sausage	76.8–95.4 79.6–96.9 82.6–98.3 80.6–98.6	LC-MS/MS	16
Estrogens, androgens, progestogens and glucocorticoids	28	Oasis HLB SPE	1 L/0.5 g	Surface water Influents Effluents Sludge	90.6–119.0 44.0–200 60.7–123 62.6–138	RRLC-MS/MS	20
Glucocorticoids, progesterones and mineralocorticoids	5	Liquid–liquid extraction	0.25 mL	Serum plasma	>95.0	LC-DMS-MS/MS	44
Thyroid hormones (T3 and T4)	2	OPT polymer SPE	0.05 mL	Plasma	82–105.0	LC-MS/MS	45
Estrogens, androgens, progesterones, adrenocortical hormones and industrial chemicals	31	MCX SPE	1000 mL	Water	84.4–103.0	LC-MS/MS	22
Estrogens, androgens, progesterones and corticoids	18	C18 SPE	1 g	Antler velvet	62.1–104.0	GC-MS/MS	46
Estrogens and industrial chemicals (preservatives, flame retardants and others)	19	MSPD	0.5	Fish	40.0–103.0	UHPLC-MS/MS	34

[a] MSPD means matrix solid-phase dispersion.

The results showed the applicability and stability of the developed method. In addition, the performance of the chosen procedure was evaluated for linear range, precision, limit of detection (LOD) and limit of quantitation (LOQ) (Table 3). The LOD and LOQ were determined as the lowest concentration tested in which the analyte gave a signal-to-noise (S/N) ratio of ≥3 and ≥10, respectively. The LOQs for the target analytes in fish and water were 0.01–30.12 ng g^{-1} and 0.01–2.56 ng mL^{-1}, respectively. These higher LOQs of the compounds in water samples, compared with those in fish samples, were probably caused by the increased chromatographic signal noise due to some interferents existing in fish.

An extensive study of the matrix effect and recovery was carried out using spiked samples of zebrafish and water. The matrix effect (ME) was calculated by the ratio between the slope of matrix-matched standard curves and the slope of standard solution curves, and then expressed as %. In this way, the ratio >100% indicates a positive matrix effect (enhancement of the signal) and the value <100% corresponds to a negative matrix effect (suppression of the signal).[42] ME values (%) are presented in Table 3. The results indicate that the ME was observed for all of the compounds except AN (77.9%), P4 (74.4%) and A (73.6%) in fish, for which no matrix effect was determined (80–120%). Thus, isotope-labeled internal standards were utilized for

Table 5 The LODs for EDCs in our present study compared with references

	LOD from our present study (ng mL^{-1} or ng g^{-1})					
Compounds	Fish	Water	Matrix sample	LOD (ng g^{-1})	Detection method	References
EST	9.04	0.31	Beef	0.03	LC-MS/MS	43
E2	5.17	0.98	Beef	0.05	LC-MS/MS	43
EE2	2.26	0.87	Bovine milk	0.09 ng mL^{-1}	LC-MS/MS	21
E1	0.96	0.24	Beef	0.02	LC-MS/MS	43
DES	0.65	0.05	Muscle	0.01	LC-MS/MS	47
			Kidney	0.03		
TB	0.30	0.04	Bovine milk	0.08 ng mL^{-1}	LC-MS/MS	21
BOL	0.12	0.01	Bovine bile	0.44 ng mL^{-1}	LC-MS/MS	48
19-NT	0.73	0.03	Antler velvet	0.8	GC-MS/MS	46
T	0.31	0.01	Beef	0.004	LC-MS/MS	43
DHEA	3.01	0.31	Beef	0.16	LC-MS/MS	43
MT	0.51	0.07	River water	0.2 ng L^{-1}	LC-MS/MS	49
ST	0.07	0.01	Chicken	0.01	LC-MS/MS	16
			Pork			
			Beef			
			Sausage			
AN	0.30	0.07	Chicken	0.01	LC-MS/MS	16
			Pork			
			Beef			
			Sausage			
Δ5-17-OHP	0.09	0.15	Plasma	1.25 ng mL^{-1}	LC-MS/MS	50
17-OHP	0.14	0.09	Chicken	0.16	LC-MS/MS	16
			Pork			
			Beef			
			Sausage			
P4	0.32	0.02	Chicken	0.01	LC-MS/MS	16
			Pork			
			Beef			
			Sausage			
Cortisone	0.09	0.13	Bovine bile	0.15 ng mL^{-1}	LC-MS/MS	48
Hd	0.21	0.20	Chicken	0.05	LC-MS/MS	16
			Pork			
			Beef			
			Sausage			
Cortexolone	0.03	0.03	Serum plasma	0.05 ng mL^{-1}	LC-DMS-MS/MS	44
Corticosterone	0.10	0.01	Serum plasma	0.03 ng mL^{-1}	LC-DMS-MS/MS	44
Dex	0.50	0.03	Bovine bile	0.14 ng mL^{-1}	LC-MS/MS	48
B	0.26	<0.01	Muscle	0.01	LC-MS/MS	47
			Kidney	0.03		
A	0.57	0.28	Plasma	0.5 ng mL^{-1}	LC-MS/MS	51
21-OHP	0.07	0.02	Serum plasma	0.1 ng mL^{-1}	LC-DMS-MS/MS	44
T3	0.14	0.12	Plasma	<0.24 ng mL^{-1}	LC-MS/MS	45
T4	0.41	0.14	Plasma	<0.42 ng mL^{-1}	LC-MS/MS	45

evaluating the matrix effect and assay reliability when the samples contained endogenous target analytes. In this study, P4-d_9, ST-d_3, E2-d_3 and EST-d_3 were used for each group of similar target substances.

3.6 Comparison with other published methods

The analytical parameters of the methods for the determination of EDCs with references are summarized in Table 4. Most methods in references are only limited to the same class (*e.g.* T3 and T4) or a few classes of EDCs, and were used to analyze less than twenty compounds. Compared to previous studies, the present study can simultaneously analyze 26 EDCs, all of which belong to six types (estrogens, androgens, progesterones, glucocorticoids, mineralocorticoids and thyroid hormones). Besides, T3 and T4 were to be detected for the first time with other EDCs in fish and water. Zhao *et al.*[18] used three SPE cartridges (C18, Si and NH_2) for the enrichment and separation of compounds, successively, whereas a single HLB cartridge could simultaneously clean up a few kinds of EDCs from various matrices, such as water and fish.

The LODs of the EDCs from references are also listed in Table 5. From the table, we can see that most compounds tested in this work had relatively low LODs than others in the references, and some were similar to previous studies. The LODs of 0.01 and 0.03 ng mL^{-1} (or ng g^{-1}), respectively, were achieved for BOL and 19-NT in our present work, which were 20-fold lower than those in the references. Furthermore, the recoveries of EDCs from the influent mentioned in Table 5 were 44.0–200%, and were 62.6–138% for the sludge,[20] which indicated that the method could not meet the expected requirement. And the same case was observed in fish with poor recoveries ranging from 40–103%, especially for E1.[34] In our work, the recoveries of all the EDCs ranged from 70.3% to 118.8% and this showed a better performance on purification and enrichment of multi-residue hormones than other methods described above.

3.7 Sample analysis

The method was applied to analyze EDCs in water and fish (five samples for each matrix). Zebrafish were obtained from a local fish market (Hangzhou, China) and water samples were from tap-water in the laboratory. The results indicated that P4 and TB were only observed in zebrafish samples at 5.73 ± 0.21 and 7.45 ± 0.34 ng g^{-1}, respectively, possibly because of small individual and low content of other EDCs for zebrafish. There was no target analyte detected in tap-water samples. Among these compounds found in real samples, two industrial compounds including STD and TB were also the key EDCs in aquatic environments.

3.8 Suitability of the method for milk samples

In order to explore the suitability of the developed method to other matrices, spiking experiments on milk samples (purchased from a local supermarket) were performed. The recoveries and RSDs of each EDC at various spiking levels are also summarized in Table S1.† As shown in Table S1,† the spiking recoveries of the 26 analytes for the milk samples were between 69.1% and 120.5% with RSDs in the range of 1.5–15.0% at all spiking levels, and the LODs were from 0.04 to 4.44 ng mL^{-1}, which were similar to those of the spiking experiments on water and fish samples. These indicated that LODs of some compounds (*e.g.* DHEA, TB, BOL, T, P4, A and E1) were lower compared to the developed multi-methods of the previous studies.[19,21] And the proposed method was applied to the analysis of milk samples (six samples for each matrix) and fresh milk was purchased from a local supermarket (Hangzhou, China). It was found that Hd, AN, STD, P4, 17-OHP and E2 were detected in fresh milk at 3.45 ± 0.13, 5.19 ± 0.17, 2.64 ± 0.08, 11.41 ± 0.42, 0.21 ± 0.03 and 1.5 ± 0.02 ng g^{-1}, respectively. The findings were also found from other papers reported in the literature.[17,19,21] Thus, the results indicated that the developed method was also suitable to determine EDCs in milk samples.

4 Conclusion

A method for the simultaneous detection of 26 EDCs in fish and water samples was developed by a modified QuEChERS-SPE-UHPLC-MS/MS method. All of the parameters involved in QuEChERS extraction and the SPE clean-up step, such as the SPE type, eluting solvent and pH, have been optimized to achieve maximum recoveries and minimum matrix effects. Compared with other methods for determining hormones in previous studies, the present method showed the numbers and classes of analytes (26 hormones, 6 classes) were more. Further cleanup using one single HLB SPE cartridge was effective in minimizing the matrix effect, which made the whole clean-up step simpler, quicker and more economical. Excellent linearity, precision, accuracy and satisfactory recoveries were obtained. The LODs of this method were similar to those obtained by the previously reported methods, while some (*e.g.* BOL and 19-NT) of them were relatively lower. The described method was successfully applied to hormone analysis in real samples, and two hormones (P4 and TB) have been determined in zebrafish samples with concentrations at 5.73 ± 0.21 and 7.45 ± 0.34 ng g^{-1}, respectively. The results of the subsequent experiment also indicated that the developed method is applicable to milk samples. Therefore, the developed method can be regarded as an alternative method to perform detection of natural and synthetic hormones, and it can also facilitate further studies in the investigation of EDCs in aquatic toxicology.

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