Journal of Chromatography A, 1218 (2011) 1367–1378

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry

Shan Liu, Guang-Guo Ying*, Jian-Liang Zhao, Feng Chen, Bin Yang, Li-Jun Zhou, Hua-jie Lai

State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, 511 Kehua Street, Tianhe, Guangzhou 510640, China

ARTICLE INFO

Article history:
Received 28 October 2010
Received in revised form
30 December 2010
Accepted 5 January 2011
Available online 12 January 2011

Keywords:
Steroids
Surface water
Wastewater
Sludge
RRLC–MS/MS

ABSTRACT

A sensitive rapid resolution liquid chromatography–tandem mass spectrometry (RRLC–MS/MS) method, combined with solid-phase extraction, ultrasonic extraction and silica gel cartridge cleanup, was developed for 28 steroids including 4 estrogens (estrone (E1), 17β-estradiol (E2), 17α-ethynyl estradiol (EE2), diethylstilbestrol (DES)), 14 androgens (androsta-1,4-diene-3,17-dione (ADD), 17α-trenbolone, 17β-trenbolone, 4-androstene-3,17-dione, 19-nortestosterone, 17β-boldenone, 17α-boldenone, testosterone (T), epi-androsterone (EADR), methyltestosterone (MT), 4-hydroxy-androst-4-ene-17-dione (4-OHA), 5α-dihydrotestosterone (5α-DHT), androsterone (ADR), stanozolol (S)), 5 progestagens (progesterone (P), ethynyl testosterone (ET), 19-norethindrone, norgestrel, medroxyprogesterone (MP)), and 5 glucocorticoids (cortisol, cortisone, prednisone, prednisolone, dexamethasone) in surface water, wastewater and sludge samples. The recoveries of surface water, influents, effluents and sludge samples were 90.6–119.0% (except 5α-DHT was 143%), 44.0–200%, 60.7–123% and 62.6–138%, respectively. The method detection limits for the 28 analytes in surface water, influents, effluents and freeze-dried sludge samples were 0.01–0.24 ng/L, 0.02–1.44 ng/L, 0.01–0.49 ng/L and 0.08–2.06 ng/g, respectively. This method was applied in the determination of the residual steroidal hormones in two surface water of Danshui River, 12 wastewater and 8 sludge samples from two wastewater treatment plants (Meihu and Huiyang WWTPs) in Guangdong (China). Ten analytes were detected in surface water samples with concentrations ranging between 0.4 ng/L (17β-boldenone) and 55.3 ng/L (5α-DHT); twenty analytes in the wastewater samples with concentrations ranging between 0.3 ng/L (P) and 621 ng/L (5α-DHT); and 12 analytes in the sludge samples with concentrations ranging between 1.6 ng/g (E1) and 372 ng/g (EADR).

1. Introduction

Endocrine disrupting chemicals (EDCs) in the environment have received a worldwide attention in recent years. It is reported that EDCs could interfere with reproduction and development, induce hermaphroditism of aquatic organisms [1], and increase the possibility of breast and testicular cancers in humans [2]. Natural and synthetic steroids have been regarded as the most important members of EDCs which could cause adverse effects on aquatic organism at the low ng/L level [3,4]. In addition to excretion of natural steroids by human and animals, synthetic steroids have also been widely used in our daily life and livestock industry for various purposes, such as contraception, human and veterinary therapy, and growth promoters. Those steroids and their metabolites are constantly discharged into environment due to their incomplete removal in wastewater treatment plants or direct excretion and discharge. Therefore, it is necessary to develop a sensitive and reliable method to analyze different classes of steroid compounds in surface water, wastewater and sludge samples in order to assess their potential environmental impact.

Plenty of methods for analysis of steroids by gas chromatography–mass spectrometry (GC–MS) have been developed due to its high separation and good identification capabilities [5]. However, GC–MS methods for steroids have some limitations, for example, requirement of derivatization and conversion problems during instrumental analysis [6–9]. One or several steps of derivatization are required in these GC–MS methods, and the derivatization steps for different classes of steroids are often complicated [7,10–12]. Several studies showed that the derivatization of 17α-ethynyl estradiol (EE2) with some silylation reagents could produce two derivatives [8,9], and similar reactions are likely to happen to other steroids owning an ethinyl group at the same

* Corresponding author. Tel.: +86 20 85290200; fax: +86 20 85290200.
E-mail address: guangguo.ying@gmail.com (G.-G. Ying).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.01.014

Table 1
Details of the estrogens and their multiple reaction monitoring (MRM) parameters in RRLC–MS/MS under negative ionization mode.

Compound	Abbreviation	Supplier	M.W.[b]	CAS	R.T.[c]	Precursor ion	Product ions	Fragmentor (V)	Collision energy (V)
Estrone-2,4,16,16-d4 (I.S.[a])	E1-d4	Cambridge	274.4	53866-34-5	4.638	273	147.2	168	37
Estrone	E1	Riedel-de Haen	270.4	53-16-7	4.643	269	145.1	148	33
							143.1	148	57
17β-Estradiol-2,4,16,16-d4 (I.S.)	E2-d4	CDN	276.4	66789-03-5	3.713	275	187.0	219	25
17β-Estradiol	E2	Dr.Ehrenstorfer	272.4	50-28-2	3.719	271	183.0	204	33
							145.0	204	30
17α-Ethynyl estradiol	EE2	Dr.Ehrenstorfer	296.4	57-63-6	4.170	295	159.0	170	34
							145.0	170	38
Diethylstilbestrol	DES	Riedel-de Haen	268.4	56-53-1	4.738	267	251.1	163	17
							237.1	163	21

[a] Internal standard.
[b] Molecular weight.
[c] Retention time (min).

position as EE2, such as some synthetic progestagens. Moreover, some steroids, such as stanozolol (an androgen), are difficult to be derivatized [13].

Fortunately, liquid chromatography–tandem mass spectrometry (LC–MS/MS) can offer an alternate choice for analysis of steroids due to recent rapid development in LC–MS/MS instrument systems. It has some advantages such as reduced analytical time and no derivatization steps. Due to its selectivity, sensitivity, simplicity and analytical throughput, LC–MS/MS has increasingly been applied to analyze different classes of steroids in wastewater and surface water samples [14–16]. However, so far few methods have been developed to simultaneously extract and analyze four classes of steroids (estrogens, androgens, progestagens and glucocorticoids) in complex environmental samples (surface water, wastewater and sludge).

The objective of this study was to develop a sensitive method for simultaneous extraction of 28 steroids including natural and synthetic estrogens, androgens, progestagens and glucocorticoids by using solid-phase extraction (SPE) and silica gel cleanup for surface water and wastewater samples, and by using ultrasonic extraction and silica gel cleanup for sludge samples, followed with analysis by rapid resolution liquid chromatography–tandem mass spectrometry (RRLC–MS/MS). This is a pioneering study that uses ultrasonic extraction to extract simultaneously these four classes of steroids in sludge samples. Then the developed method was applied to determine these steroid compounds in wastewater and sludge samples in two selected wastewater treatment plants (WWTPs) and surface water samples of Danshui River in Guangdong, China.

2. Materials and methods

2.1. Chemicals and materials

High purity standards of 28 steroids including 4 estrogens (estrone (E1), 17β-estradiol (E2), 17α-ethynyl estradiol (EE2), diethylstilbestrol (DES)), 14 androgens (androsta-1,4-diene-3,17-dione (ADD), 17α-trenbolone, 17β-trenbolone, 4-androstene-3,17-dione, 19-nortestoserone, 17β-boldenone, 17α-boldenone, testosterone (T), epi-androsterone (EADR), methyltestosterone (MT), 4-hydroxy-androst-4-ene-17-dione (4-OHA), 5α-dihydrotestosterone (5α-DHT), androsterone (ADR), stanozolol (S)), 5 progestagens (progesterone (P), ethynyl testosterone (ET), 19-norethindrone, norgestrel, medroxyprogesterone (MP)), and 5 glucocorticoids (cortisol, cortisone, prednisone, prednisolone, dexamethasone) and their internal standards estrone-2,4,16,16-d4 (E1-d4), 17β-estradiol-2,4,16,16-d4 (E2-d4), testosterone-16,16,17-d3 (T-d3), stanozolol-d3 (S-d3), progesterone-d9 (P-d9), cortisol-d2(CRL-d2) were purchased from Dr. Ehrenstorfer GmbH (Germany), Supelco (USA), Riedel-de Haën (RDH, Germany), Sigma–Aldrich (USA), Cambridge Isotope Laboratories Incorporation (Massachusetts, USA), CDN Isotopes (Quebec, Canada), TCR (North York, Canada), Cerilliant (USA), ACROS and Sigma (St. Louis, MO, USA) (Tables 1 and 2). All reagents of HPLC grade used for sample processing and analysis (methanol (ME), acetonitrile, ethyl acetate (EA), hexane and dichloromethane (DCM)) were obtained from Merck Corporation (Shanghai, China) or CNW Technologies (Dusseldorf, Germany). Formic acid and acetic acid were obtained from Tedia company (Tedia, USA) and Sigma–Aldrich (St. Louis, USA). Oasis HLB cartridges (N-vinylpyrrolidone-m-divinylbenzene copolymer, 500 mg, 6 mL) were obtained from Waters Corporation (Milford, MA, USA), whereas Supelclean ENVI-18 cartridges (500 mg, 6 mL) were purchased from Supelco Corporation. Glass fiber filters (GF/F, pore size 0.7 μm) were supplied by Whatman (Maidstone, England) and pyrolyzed at 450 °C for 4 h prior to use. Neutral silica gel (100–200 mesh, Qingdao, China) was Soxhlet extracted with dichloromethane for 48 h and baked at 160 °C for 16 h prior to use. Anhydrous sodium sulfate was baked at 450 °C and stored in a sealed desiccator. HPLC grade water was obtained from a Milli-Q water purification system (Millipore, Watford). Stock solutions of chemicals (100 mg/L) were prepared in methanol and stored at −18 °C for later use. Working standard solutions were prepared weekly. All glassware was hand-washed with detergent and tap water, rinsed with HPLC grade water, and baked at 450 °C for at least 4 h before use.

2.2. Sample collection

The surface water samples were collected from Liuxi Reservoir and Danshui River. Liuxi Reservoir is located in Conghua, Guangzhou which is one of the most important drinking water sources in Guangdong province. The grab water samples from the reservoir were only used for recovery tests. Danshui River is located in Huiyang, and receives the discharge of effluents from Huiyang WWTP. Wastewater and sludge samples were collected from Huiyang and Meihu WWTPs. Both Huiyang and Meihu WWTPs are located in Guangdong province (China) and operated with primary, anoxic, anaerobic, aerobic biological and secondary treatment processes. Domestic wastewater is the main source of the incoming raw sewage water in the two WWTPs. The surface water samples of Danshui River, and wastewater and sludge samples from the two WWTPs were collected in 1 L amber glass bottles at 6 p.m., 8 a.m. and 3 p.m. in two consecutive days on May 24–25, 2010, and mixed the three time point samples together as a composite sample. Table S1 shows the basic information of these two WWTPs. Three parallel samples were collected from each site. For water samples, about 50 mL of methanol was added to each bottle (1 L) and the pH was adjusted to 3 using 4 M H_2SO_4 in the field. One gram of sodium azide was added to each liter of sludge samples to suppress microbial activity. Water samples were transported back to laboratory and stored in the dark at 4 °C, and then processed

Table 2
Details of the androgens, progestagens, glucocorticoids, and their MRM parameters in RRLC–MS/MS under positive ionization mode.

Compound[a]	Supplier	M.W.[b]	CAS	R.T.[c]	Precursor ion	Product ions	Fragmentor (V)	Collision energy (V)
Glucocorticoids								
Prednisone	Dr. Ehrenstorfer	358.4	53-03-2	3.131	359.2	147	120	28
						91	120	72
Cortisone	Sigma	360.5	53-06-5	3.228	361.2	163	155	20
						105	155	48
Cortisol-d2 (I.S.)	CDN isotopes	364.4	79037-25-5	3.695	365.2	122	165	24
						91.1	165	76
Cortisol	Dr. Ehrenstorfer	362.5	50-23-7	3.701	363.2	121	170	24
						91	170	72
Prednisolone	Dr. Ehrenstorfer	360.4	50-24-8	3.729	361.2	343.1	135	4
						147	135	20
Dexamethasone	Dr. Ehrenstorfer	392.5	50-02-2	4.760	393.2	147	125	28
						91	125	72
Androgens								
ADD	TCR	284.4	897-06-3	5.438	285.2	121.1	105	21
						77.1	105	61
17α-Trenbolone	CERILLIANT	270.4	80657-17-6	6.405	271.2	253.2	150	20
						115	150	96
17β-Trenbolone	Dr. Ehrenstorfer	270.4	10161-33-8	6.411	271.2	165.1	140	69
						128	140	69
4-Androstene-3,17-dione	Dr. Ehrenstorfer	286.4	63-05-8	7.129	287.2	109.1	135	25
						97.1	135	21
19-Nortestoserone	Dr. Ehrenstorfer	274.4	434-22-0	7.238	275.2	109.1	130	45
						55	130	61
17β-Boldenone	Dr. Ehrenstorfer	286.4	846-48-0	6.956	287.2	135.1	90	9
						121.1	90	21
17α-Boldenone	CERILLIANT	286.4	27833-18-7	7.601	287.2	269.2	85	5
						121.1	85	25
Testosterone	Dr. Ehrenstorfer	288.4	58-22-0	8.565	289.2	109.1	135	25
						97.1	135	21
Testosterone-16,16,17-d3 (I.S.)	CERILLIANT	291.4	77546-39-5	8.501	292.2	109.1	135	25
						97.1	135	29
Epi-androsterone	ACROS	290.4	481-29-8	9.820	291.2	273.2	90	5
						175.2	90	9
Methyltestosterone	Dr. Ehrenstorfer	302.4	58-18-4	10.253	303.2	109.1	140	29
						97.1	140	25
4-OHA	TRC	302.4	566-48-3	10.256	303.2	257.2	110	13
						55.1	110	53
5α-DHT	Dr. Ehrenstorfer	290.4	521-18-6	10.458	291.2	273.2	115	9
						255.2	115	13
Androsterone	Dr. Ehrenstorfer	290.4	53-41-8	12.114	291.2	273.2	90	5
						255.2	90	13
Stanozolol-d3 (I.S.)	CERILLIANT	331.5	88247-87-4	13.202	332.3	81.1	220	53
						54.1	220	89
Stanozolol	Dr. Ehrenstorfer	328.5	10418-03-8	13.259	329.3	81.1	215	73
						54.1	215	100
Progestagens								
19-Norethindrone	TRC	298.4	68-22-4	7.260	299.2	109.1	130	29
						77.1	130	73
Ethynyl testosterone	Dr. Ehrenstorfer	312.4	434-03-7	8.514	313.2	109.1	135	25
						97.1	135	21
Norgestrel	Sigma	312.4	6533-00-2	9.579	313.2	91.1	135	61
						77.1	135	77
Medroxyprogesterone	Dr. Ehrenstorfer	344.5	520-85-4	10.640	345.2	123.1	145	25
						97.1	145	29
Progesterone-d9 (I.S.)	TCR	323.5	15775-74-3	12.527	324.3	113.1	125	29
						100.1	125	25
Progesterone	Dr. Ehrenstorfer	314.4	57-83-0	12.715	315.2	109.1	130	25
						97.1	130	21

[a] ADD, androsta-1,4-diene-3,17-dione; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; 5α-DHT, 5α-dihydrotestosterone; I.S., internal standard.
[b] Molecular weight.
[c] Retention time (min).

within 48 h. Liquid sludge samples were centrifuged, freeze dried, crushed and homogenized. Dried sludge (0.5 g) was prepared for each extraction.

2.3. Sample extraction and cleanup

2.3.1. Water sample extraction

Solid phase extraction (SPE) was used to extract water samples (Fig. 1). One liter of water samples was filtered through glass fiber filters (Whatman GF/F, 0.7 μm effective pore size, UK). Exactly 100 μL each of 1 mg/L of E1-d4, E2-d4, T-d3, S-d3, CRL-d2 and P-d9 was added to each sample as the internal standards. Solid phase extraction method for water samples was developed by testing with two SPE cartridges (Oasis HLB and Superclean C18), four elution solvents (ethyl acetate, dichloromethane, methanol and methanol/dichloromethane (7:5, v/v)), and two pH values (7 and 3). The optimized SPE method was described as follows. Solid phase extraction cartridges (Oasis HLB, 6 mL and 500 mg each) were pre-

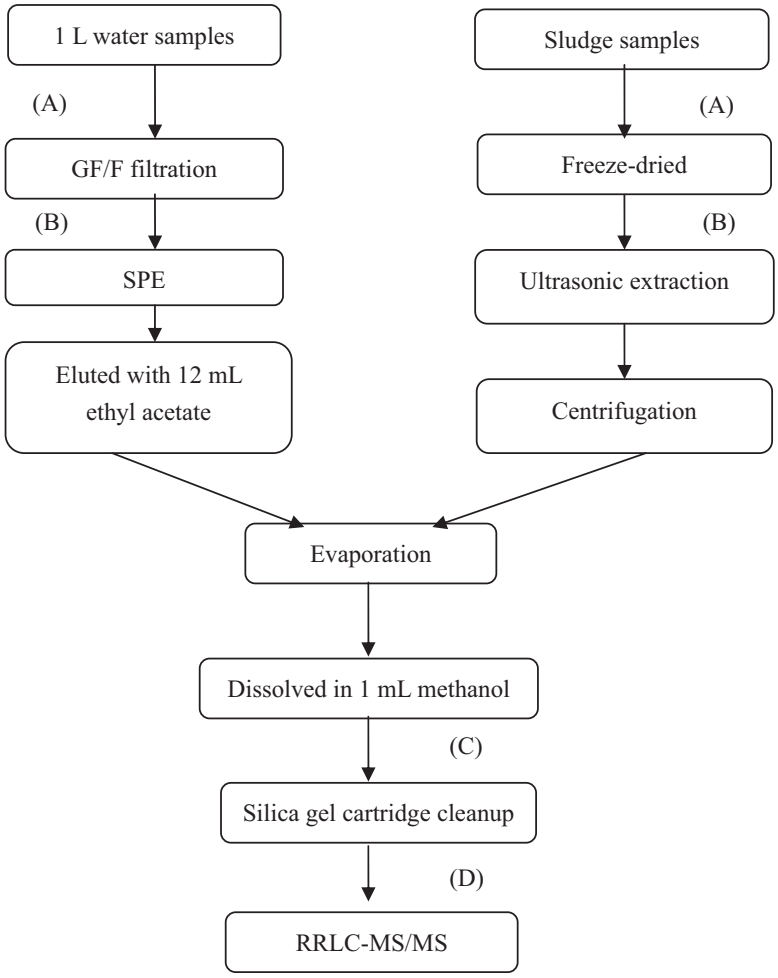

Fig. 1. Schematic diagram showing each step of water and sludge samples preparation procedure. (A) 50 mL of methanol was added to each 1 L water samples and the pH was adjusted to 3 using 4 M H_2SO_4, 1 g of sodium azide was added to each liter of sludge samples to suppress microbial activity. (B) 100 μL each of 1 mg/L of E1-d4, E2-d4, T-d3, S-d3, CRL-d2 and P-d9 was added to each sample as the internal standards. (C) Each extract (240 μL) was added to the silica cartridge for cleanup. (D) The target compounds were eluted with 6 mL of ethyl acetate/methanol (90:10, v/v). The eluate was then dried and reconstituted in 240 μL. Before analysis, 100 μL of that concentrated solution was dried and reconstituted in a buffer for the RRLC/MSMS analysis. For negative mode, the buffer was methanol/water (50:50, v/v), whereas for positive mode, the buffer was methanol/water–0.01% formic acid (60:40, v/v).

conditioned each with 10 mL of methanol followed by 10 mL of HPLC grade water. The filtered water samples passed through the SPE cartridges at a flow rate of 5–10 mL/min. The sample bottle was rinsed twice with two aliquots of 50 mL of 5% (v/v) methanol in HPLC grade water, which passed through the cartridge. Then the cartridges were dried under the vacuum for 2 h, and the target compounds were eluted from the cartridges using 12 mL of ethyl acetate. The extracts were dried and re-dissolved in 1 mL of methanol. Each final extract was then filtered through a 0.22 μm membrane filter into a 2 mL amber glass vial for further cleanup.

2.3.2. Sludge sample extraction

Sludge sample extraction method was developed by optimizing sludge weights (0.5, 1.0 and 2.0 g), and different extraction solvents (ethyl acetate, ethyl acetate/methanol (9:1 and 8:2, v/v), acetonitrile, formic acid/methanol (1:50, v/v) and methanol) spiked at a concentration of 100 ng/g (Fig. 1). The final optimized method for simultaneous extraction of 28 steroids in sludge used 0.5 g of sludge sample and ethyl acetate as the extraction solvent with the detailed procedure given as follows. Three parallel freeze-dried sludge samples (0.5 g), spiked with 100 ng of E1-d4, E2-d4, T-d3, S-d3, CRL-d2 and P-d9 as internal standards, were put into 30 mL glass centrifuge tubes. To volatilize the solvent from the sludge samples, the tubes were put into the fume hood for 4 h with foil loosely caped, manually mixed well, and then kept in 4 °C overnight. The sludge sample was extracted with 10 mL of ethyl acetate in an ultrasonic bath for 15 min and then centrifuged at 3500 rpm for 10 min. The supernatant was transferred into 100 mL pear-shaped flask by a glass pipette. The extraction process was repeated twice using 10 mL and 5 mL of the above extraction solution. Then all 25 mL extract from each sample was combined, evaporated to dryness via rotary evaporation, redissolved in 1 mL of methanol, and passed through 0.22 μm filter for further cleanup.

2.3.3. Cleanup

Normally, to reduce matrix interference, further cleanup of wastewater and sludge samples is required [17,18]. In this study, self-made silica gel cartridge (18 cm × 1 cm i.d.), which had been extracted by dichloromethane for 48 h, was used for further cleanup. The cleanup step was optimized by testing different elution solvents, ethyl acetate, ethyl acetate/methanol (95:5, 90:10, 85:15 and 80:20, v/v), and the optimized cleanup procedure is given as follows. The glass cartridge (self-made) was filled with glass wool (CNW), 1.0 g silica gel and 0.5 cm of anhydrous sodium sulfate from bottom to top. Each extract (240 μL) was added to the silica cartridge, which was preconditioned with 5 mL of methanol, 5 mL of ethyl acetate/methanol (90:10, v/v), and 5 mL of hexane. After the cartridge was rinsed with 6 mL of hexane, the target compounds

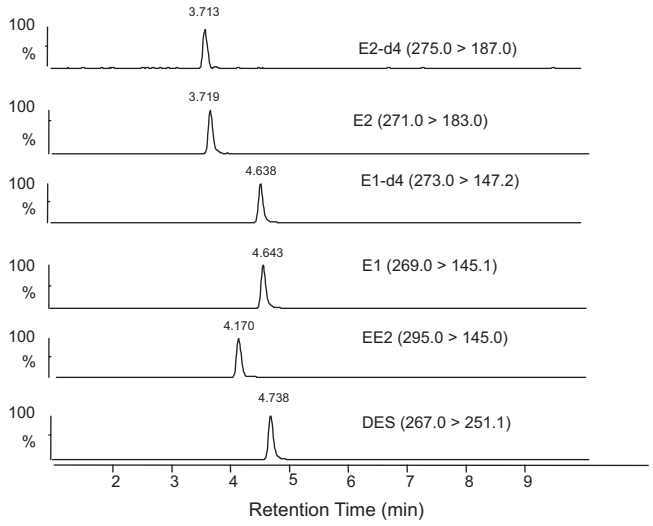

Fig. 2. Extracted ion chromatograms (EIC) of the quantitative ions for estrogens in standard solution at the concentration each of 100 ng/L. Conditions: RRLC–MS/MS with negative ionization in MRM mode. E2-d4, 17β-estradiol-d4; E2, 17β-estradiol; E1-d4, estrone-d4; E1, estrone; EE2, 17α-ethynyl estradiol; DES, diethylstilbestrol.

were eluted with 6 mL of ethyl acetate/methanol (90:10, v/v). The eluate was then dried and reconstituted in 240 μL. Before analysis, 100 μL of that concentrated solution was dried and reconstituted in a buffer for the RRLC/MSMS analysis. For negative mode, the buffer was methanol/water (50:50, v/v), whereas for positive mode, the buffer was methanol/water–0.01% formic acid (60:40, v/v).

2.4. Instrumental analysis

The target compounds were analyzed by RRLC–MS/MS with electrospray ionization (ESI). Liquid chromatography was performed on an Agilent 1200 series RRLC system (Agilent Technologies) equipped with a degasser, a binary pump, an auto sampler and a column oven. The chromatographic separation was performed on an Agilent Zorbax SB-C18 (100 mm × 3 mm, 1.8 μm) column with its corresponding pre-column filter (2.1 mm, 0.2 μm). The column oven temperature was set to 40 °C and the injection volume was 10 μL. Two gradient elution programs were applied for two groups of steroids (Group I: estrogens; Group II: androgens, progestagens, and glucocorticoids), with a flow rate at 0.3 mL/min (Group I) and 0.35 mL/min (Group II), respectively. Mass spectrometry was performed using an Agilent 6460 Triple Quadrupole detector which was operated with ESI in both negative and positive modes (Agilent Corporation, USA). The quantitative analysis of the target compounds was performed in multiple reaction monitoring (MRM) mode. Nitrogen gas was used as the drying and collision gas. Multiple reaction monitoring (MRM) parameters for the target compounds and internal standards are listed in Tables 1 and 2. The extracted ion chromatograms (EIC) of the quantitative ions for the steroid compounds in the standard solution at the concentration of 100 ng/L each are shown in Figs. 1 and 2.

LC parameters and ESI mode parameters for estrogens (Group I) and androgens, progestagens, and glucocorticoids (Group II) are given in Table 3. Data acquisition for Group II was divided into three retention time periods (2.5–4.4, 4.4–11.6, and 11.6–15 min) to ensure that enough dwell time was spent on each transition (Fig. 3).

2.5. Data analysis

The analytes were identified by comparing the retention times (within 2%) and the ratios (within 20%) of the two selected precursor–product ion transitions with those of the standards.

Table 3
Details of LC and MS operating parameters for these two groups of steroids.

Compound	LC						MS					
	LC gradient program			Flow rate (mL/min)	Column temperature (°C)	Max pressure (bar)	Gas temperature (°C)	Gas flow (mL/min)	Nebulizer (psi)	Sheath gas flow (L/min)	Sheath gas temperature (°C)	Nozzle voltage (V)	Capillary voltage (V)
	Time (min)	Solvent A[a] (%)	Solvent B[b] (%)										
Group I	0	50	50	0.3	40	400	350	8	50	12	350	−2000	3500
	10	0	100										
Group II	0	40	60	0.35	40	400	350	3	40	12	350	2000	3500
	15	20	80										
	15.5	40	60										

[a] Solvent A is water for Group I, and formic acid (0.01%, v/v) for Group II.
[b] Solvent B is acetonitrile for Group I and methanol for Group II.

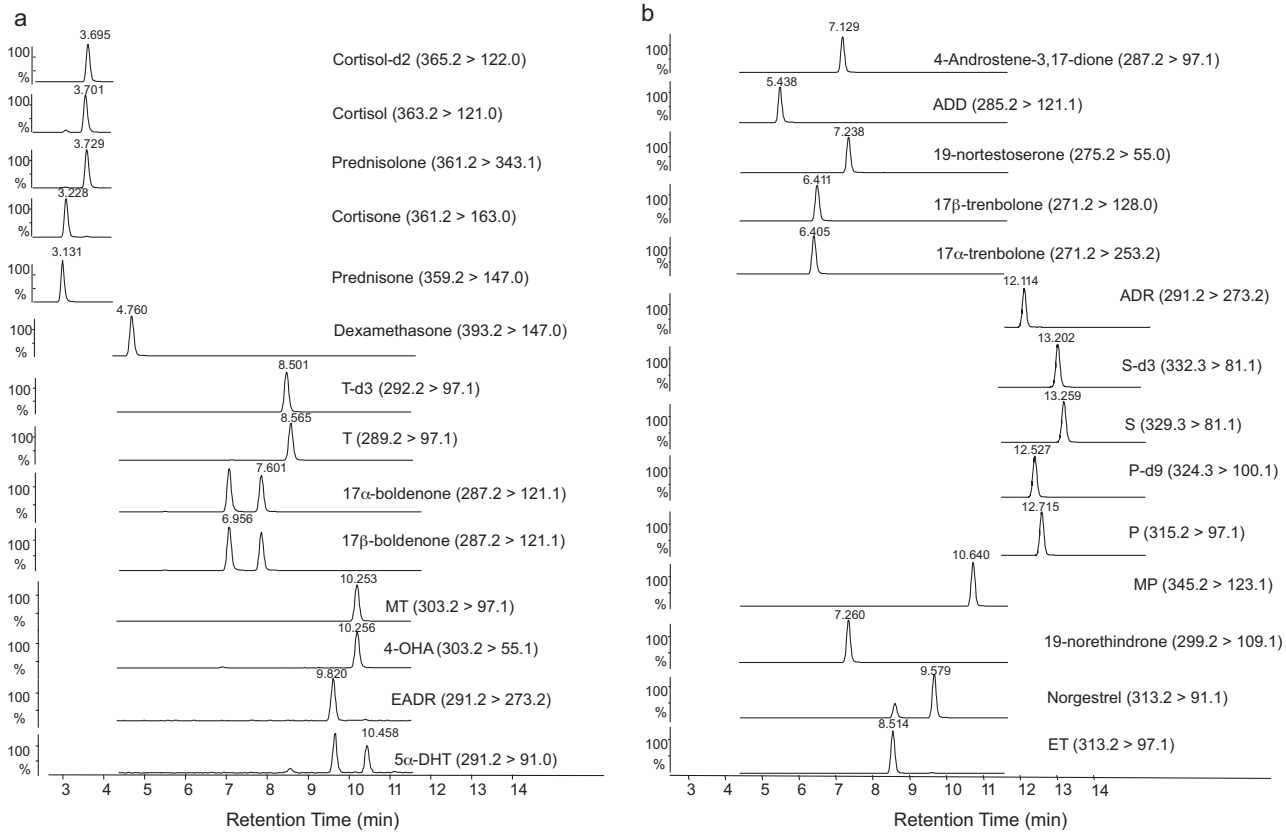

Fig. 3. (A) Extracted ion chromatograms (EIC) of the quantitative ions for glucocorticoids, androgens and progestagens in standard solution at the concentration each of 100 ng/L. Conditions: RRLC–MS/MS with positive ionization in MRM mode. T-d3, testosterone-d3; T, testosterone; MT, methyltestosterone; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; EADR, epi-androsterone; 5α-DHT, 5α-dihydrotestosterone; ADD, androsta-1,4-diene-3,17-dione; S-d3, stanozolol-d3; S, stanozolol; P-d9, progesterone-d9; P, progesterone; MP, medroxyprogesterone; ET, ethynyl testosterone.(B) Extracted ion chromatograms (EIC) of the quantitative ions for glucocorticoids, androgens and progestagens in standard solution at the concentration each of 100 ng/L. Conditions: RRLC–MS/MS with positive ionization in MRM mode. T-d3, testosterone-d3; T, testosterone; MT, methyltestosterone; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; ADR, androsterone; 5α-DHT, 5α-dihydrotestosterone; ADD, androsta-1,4-diene-3,17-dione; S-d3, stanozolol-d3; S, stanozolol; P-d9, progesterone-d9; P, progesterone; MP, medroxyprogesterone; ET, ethynyl testosterone.

Quantification of the target compounds was performed using internal standard method. Laboratory blanks were also analyzed along with the samples to assess potential sample contamination. Recovery experiments were done by spiking the standard solutions to influent, effluent, surface water and sludge samples. Data acquisition was performed under Agilent Mass Hunter B 02.01 software.

3. Results and discussion

3.1. Optimization of SPE conditions

SPE was used to extract the target compounds in water samples after removal of particles by filtration. Different pH, SPE cartridges and elution solvents were tested during the development of the SPE method for the steroids in water. All the experiments were performed by spiking with the standard solutions of 100 ng/L to 1 L filtered Liuxi Reservoir water.

Results in Tables S2 and S3 showed that the recoveries of EADR, 5α-DHT and DES were significantly influenced by different extraction conditions using the same cartridges, while the other targets always had good recoveries, thus the recoveries of EADR, 5α-DHT and DES were the primary consideration during the optimization. As for the cartridges, the recoveries of EADR, 5α-DHT and DES were much better on HLB cartridges than on ENVI-18 cartridges. In addition, the recoveries of most analytes were out of the range of 80–120% when using ENVI-18 cartridges combined with DCM as elution solvent, regardless of pH value (3 or 7). Probably due to the low polarity of DCM, it was difficult to elute the analytes, such as glucocorticoids and some androgens with relatively high polarity, from the ENVI-18 cartridges.

Comparing those at pH 3 with pH 7, it was found that the SPE recoveries of some targets were higher at water pH 7 than pH 3, especially for EADR and 5α-DHT with the recoveries out of the range of 80–120%. Thus HLB cartridges and pH 3 were the better choice for SPE of the target compounds. As for the elution solvent for SPE, it was found that ethyl acetate gave the best recoveries at water pH 3, which were within the range of 90.6–119%, except for 5α-DHT (143%). Therefore, the optimized SPE method for the steroids in water samples was: adjusting water samples to pH 3, extracting the water samples using HLB cartridges, and eluting the target compounds with ethyl acetate.

3.2. Optimization of sludge extraction

3.2.1. Optimization of extraction solvent

Solid matrices (e.g., soil, sediment or sludge samples) are usually extracted by ultrasonic solvent extraction [19,20], microwave-assisted solvent extraction [20], pressurized liquid extraction [21], or Soxhlet extraction [22]. In this study, we chose ultrasonic solvent extraction.

All the experiments were performed by spiking 200 ng of each target compound to 2.0 g freeze-dried sludge in this section. According to the chemical properties of targets, we tested six extraction solvents, methanol, methanol/formic acid (50:1, v/v), acetonitrile, ethyl acetate, ethyl acetate/methanol (90:10, v/v), and ethyl acetate/methanol (80:20, v/v). Only 14, 15, 15, 15,

12, 8 out of 28 analytes (Table S4) fell in the recovery range of 70–120% for ethyl acetate, ethyl acetate/methanol (90:10, v/v), ethyl acetate/methanol (80:20, v/v), acetonitrile, methanol/ formic acid (50:1, v/v), methanol, respectively.

Due to its polarity, methanol or methanol/formic acid mixture had strong ability to extract a wide range of chemicals in samples, and it not only extracted the target compounds but also other polar impurities (such as pigment, and humic materials). These impurities could increase the difficulty in the cleanup step, cause matrix interferences to the analysis of the target compounds, thus reducing the precision of instrumental analysis. In addition, the other four solvents had showed similar extraction efficiencies. Considering the toxicity of solvents and simplicity of operation, ethyl acetate was chosen as the extraction solvent for sludge samples.

3.2.2. Optimization of sludge weight

By optimizing the extraction solvents, we did not achieve desirable recoveries for all target compounds due to the complexity of sludge samples that contained large amounts of other impurities. In order to reduce the interferences of the impurities, sludge weights were tested by using 0.5 g, 1.0 g, and 2.0 g of the freeze-dried sludge. All samples were spiked with 200 ng of each analyte and extracted using 25 mL ethyl acetate as above.

The results showed that 24, 24, and 14 of 28 analytes fell in the recovery range of 70–120% for 0.5 g, 1.0 g, 2.0 g of the freeze-dried sludge, respectively (Table S5). Clearly, lower sludge sample weight gave better recoveries of the target compounds. Therefore, the optimized extraction method for sludge samples chose 0.5 g freeze-dried sludge with 25 mL ethyl acetate as the extract solvent.

3.3. Optimization of cleanup

To reduce interferences of impurities in the samples, cleanup is essential before analysis by LC–MS/MS, especially for complex matrices like sludge. In several previous studies, silica gel was applied in the cleanup step [17,18,23,24]. To develop an efficient method for the purification of wastewater or sludge extracts, self-made silica gel cartridges were used in this study.

In the cleanup step, elution solvent is the most important parameter. The best elution solvent should be able to elute the target compounds, have good recoveries, and reduce other interfering compounds and matrix substances. In this optimization test, elution solvent was optimized by spiking with 100 ng of analytes to the silica gel cartridges (1.0 g), which had been preconditioned with 5 mL of methanol, 5 mL of selected elution solvent, and 5 mL of hexane. The polarities of the target compounds are very different ranging from high polarity to medium polarity: glucocorticoids ($\log K_{ow}$: 1.24–2.06), androgens ($\log K_{ow}$: 2.45–4.42), progestagens ($\log K_{ow}$: 2.97–3.51), and estrogens ($\log K_{ow}$: 2.45–5.64). On this account, five elution solvents, ethyl acetate, ethyl acetate/methanol (95:5, v/v), ethyl acetate/methanol (90:10, v/v), ethyl acetate/methanol (85:15, v/v), and ethyl acetate/methanol (80:20, v/v) were tested for use in the cleanup step.

The results (Table S6) showed that the relatively good recoveries were achieved for androgens and progestagens using the five selected elution solvents. The recoveries for glucocorticoids and estrogens varied among the different solvents. Glucocorticoids could not be eluted by ethyl acetate from the silica gel cartridges at all, suggesting that glucocorticoids needed a solvent with relatively higher polarity than ethyl acetate. It was found that ethyl acetate/methanol (90:10, v/v) as elution solvent gave the best recoveries (80.4–117%) for the target compounds except for DES with 50.9%. Thus, ethyl acetate/methanol (90:10, v/v) was selected as the elution solvent in the cleanup step.

3.4. Optimization of the mobile phase for RRLC–MS/MS

Agilent Optimizer software was used to optimize LC–MS/MS operating conditions for both positive and negative ionization modes by infusing the standard solutions (1 mg/L each). The base peak selected for quantification of the investigated estrogens corresponded to the deprotonated molecule $[M-H]^-$. Androgens, progestagens and glucocorticoids were detected by using protonated molecules $[M+H]^+$.

The instrumental conditions in the negative mode for the estrogens were further optimized from our previous study [17], using acetonitrile and pure water as the mobile phase. To optimize the operating conditions in positive mode, two organic solvents (methanol and acetonitrile) and different water phases (acetic acid and formic acid with different ratio) were tested. The best operating conditions were obtained with formic acid aqueous (0.01% formic acid, pH 3). Although better peak shapes were obtained with acetonitrile as the organic solvent, methanol was used in this study in order to have a better separation for all 24 analytes with similar structures. Thus the optimized mobile phases were: acetonitrile and pure water for analysis of estrogens (Group I) in the negative mode, and methanol and 0.01% formic acid solution for analysis of androgens, progestagens, and glucocorticoids (Group II) in the positive mode.

3.5. Matrix effect

Matrix effect is a common problem for LC–MS/MS with ESI mode [25–27], which can lead to signal suppression or enhancement and is often difficult to eliminate through cleanup procedures. Matrix effects were observed and evaluated by spiking standard solutions (100 ng/L) into the surface water, wastewater and sludge extract samples. The values of less or greater than 100% indicate signal suppression or enhancement, respectively. The results in Tables 4–6 showed that matrix components in surface water or effluent had no significant effect on signal responses of the target compounds after internal standard corrections. But matrix components in influent samples decreased signal responses of 17α-trenbolone, 17β-trenbolone, ADR, DES, with matrix effects of 41.9–68.0%, but increased signal responses of prednisone, dexamethasone and MP, with matrix effects of 132–186%. Matrix components in sludge samples also decreased signal responses of cortisone (matrix effect 67.9%), prednisolone (50.0%), ADR (69.8%), and EE2 (63.0%).

3.6. Method validation

Calibration curves were constructed for androgens from 1.0 to 1000 μg/L (standard concentration levels at 1.0, 5.0, 10, 50, 100, 200, 500, 1000 μg/L) and for other targets from 1.0 to 200 μg/L (standard concentration levels at 1, 5, 10, 50, 100 and 200 μg/L), and excellent linearity was achieved in these concentration ranges with the correlation coefficients higher than 0.99 for all validation batches.

Using the optimized extraction and instrumental methods, good recoveries were achieved for all target compounds in matrix spiked samples of surface water, wastewater and sludge samples (Tables 4–6). The limit of detection (LOD) and limit of quantitation (LOQ) for each target compound were calculated based on the signal-to-noise ratio (SNR) near the target peak. LOD is defined as three times of SNR, and LOQ is ten times of SNR. The LOQs for the target analytes in the influent, effluent, surface water, and freeze-dried sludge samples were 0.05–4.8, 0.02–1.63, 0.03–0.80 ng/L, and 0.3–6.9 ng/g, respectively (Tables 4–6). Those higher LOQs of the compounds in the influent and freeze-dried sludge samples, compared with those in the effluent and surface water, were probably

Table 4
Recoveries and detection limits of steroids in influent and effluent samples by RRLC–MS/MS.

Compound[a]	Influent[b]				LOD[c] (ng/L)	LOQ[c] (ng/L)	Effluent[b]				LOD[c] (ng/L)	LOQ[c] (ng/L)
	50 ng/L	100 ng/L	200 ng/L	Matrix%			20 ng/L	50 ng/L	100 ng/L	Matrix%		
Prednisone	158 ± 8.2	172 ± 4.9	107 ± 2.6	151 ± 2.8	0.35	1.18	104 ± 2.4	95.2 ± 5.6	99.1 ± 2.5	104 ± 1.5	0.10	0.32
Cortisone	128 ± 1.4	133 ± 5.4	72.6 ± 1.0	116 ± 3.0	0.27	0.89	75.9 ± 1.0	72.6 ± 1.8	78.4 ± 1.5	81.7 ± 0.6	0.12	0.38
Cortisol	84.6 ± 8.7	105 ± 7.8	92.9 ± 0.5	100 ± 3.5	0.41	1.37	79.0 ± 4.8	86.0 ± 2.0	102 ± 2.3	98.8 ± 2.8	0.14	0.47
Prednisolone	87.6 ± 0.5	100 ± 1.1	109 ± 2.2	101 ± 1.9	0.39	1.31	119 ± 2.8	105 ± 3.1	114 ± 4.1	111 ± 2.3	0.29	0.97
Dexamethasone	192 ± 1.8	200 ± 11.2	115 ± 2.2	186 ± 3.3	0.45	1.50	127 ± 6.0	114 ± 9.5	122 ± 4.3	126 ± 4.5	0.25	0.83
ADD	108 ± 5.2	112 ± 5.1	74.8 ± 0.2	100 ± 1.1	0.17	0.55	112 ± 4.1	102 ± 1.6	111 ± 1.2	112 ± 1.8	0.08	0.28
17α-Trenbolone	64.0 ± 3.0	67.2 ± 2.8	61.4 ± 3.0	68.0 ± 2.2	0.16	0.54	76.0 ± 4.6	71.1 ± 1.2	77.7 ± 2.0	84.1 ± 1.1	0.09	0.31
17β-Trenbolone	86.4 ± 4.9	63.2 ± 5.3	72.3 ± 4.7	57.5 ± 4.9	0.33	1.09	112 ± 5.8	99.4 ± 2.3	109 ± 0.5	113 ± 0.7	0.15	0.50
17β-Boldenone	107 ± 2.8	113 ± 1.9	104 ± 1.1	105 ± 1.2	0.18	0.60	122 ± 5.6	109 ± 3.4	114 ± 1.2	116 ± 1.7	0.11	0.38
4-Androstene-3,17-dione	101 ± 3.2	107 ± 2.8	96.9 ± 1.2	103 ± 1.4	0.28	0.95	110 ± 4.7	103 ± 1.6	112 ± 1.2	112 ± 1.6	0.11	0.37
19-Nortestoserone	84.9 ± 4.1	90.8 ± 4.0	91.6 ± 1.2	90.6 ± 1.2	0.31	1.03	105 ± 3.4	99.3 ± 2.1	103 ± 4.0	105 ± 0.5	0.31	1.03
19-Norethindrone	93.8 ± 6.4	103 ± 4.6	96.0 ± 2.4	101 ± 0.2	0.42	1.40	111 ± 3.8	97.1 ± 14.7	94.9 ± 6.5	93.2 ± 1.6	0.21	0.71
17α-Boldenone	91.6 ± 4.0	99.2 ± 6.2	126 ± 5.9	99.2 ± 3.9	0.14	0.47	128 ± 4.1	114 ± 4.0	113 ± 3.9	132 ± 3.0	0.11	0.38
Ethynyl testoserone	108 ± 3.6	116 ± 5.4	109 ± 2.8	112 ± 2.2	0.18	0.59	104 ± 3.1	92.5 ± 11.8	93.9 ± 6.8	90.1 ± 1.1	0.09	0.30
Testosterone	98.3 ± 1.0	105 ± 2.6	101 ± 0.5	100 ± 0.9	0.26	0.88	106 ± 1.5	98.6 ± 0.6	103 ± 1.7	105 ± 1.8	0.11	0.37
Norgestrel	111 ± 4.7	115 ± 5.6	105 ± 3.2	113 ± 2.0	0.31	1.03	142 ± 0.1	111 ± 10.8	100 ± 6.3	100 ± 2.5	0.03	0.10
Epi-androsterone	127 ± 5.5	133 ± 33.8	100 ± 3.4	99.2 ± 8.8	0.27	0.91	105 ± 3.6	94.9 ± 3.7	103 ± 5.8	97.9 ± 6.3	0.26	0.87
4-OHA	122 ± 2.0	92.1 ± 3.2	81.4 ± 2.1	102 ± 3.3	0.22	0.72	118 ± 4.3	103 ± 1.1	104 ± 3.4	110 ± 1.9	0.17	0.56
Methyltestoserone	92.3 ± 1.4	88.5 ± 2.4	91.0 ± 1.9	88.5 ± 0.6	0.24	0.79	95.3 ± 6.4	94.7 ± 1.5	94.2 ± 2.8	101 ± 1.8	0.07	0.24
5α-DHT	77.8 ± 1.2	130 ± 2.8	115 ± 2.3	114 ± 3.6	0.90	3.01	155 ± 0.8	102 ± 3.3	123 ± 2.8	125 ± 15.7	0.39	1.30
Medroxyprogesterone	125 ± 3.5	133 ± 5.2	127 ± 3.3	132 ± 2.9	0.12	0.40	137 ± 8.5	119 ± 10.6	117 ± 7.5	116 ± 0.8	0.04	0.15
Androsterone	101 ± 0.9	80.8 ± 14.0	26.3 ± 1.2	65.7 ± 6.6	0.82	2.72	81.6 ± 0.5	69.0 ± 4.2	80.6 ± 4.7	83.1 ± 3.6	0.40	1.33
Progesterone	104 ± 1.0	110 ± 2.2	98.1 ± 1.1	107 ± 1.2	0.09	0.29	101 ± 4.6	93.1 ± 1.8	98.6 ± 0.3	101 ± 0.6	0.08	0.27
Stanozolol	107 ± 0.7	113 ± 2.0	103 ± 0.9	109 ± 0.6	0.02	0.05	97.6 ± 1.7	91.6 ± 1.5	99.3 ± 0.6	101 ± 0.3	0.01	0.02
E2	123 ± 13.6	139 ± 31.5	115 ± 5.3	83.5 ± 1.3	1.44	4.81	121 ± 10.0	108 ± 11.3	106 ± 11.7	88.4 ± 4.2	0.29	0.95
EE2	89.1 ± 7.2	94.5 ± 7.9	76.0 ± 3.9	107 ± 2.8	0.72	2.38	74.2 ± 4.8	79.9 ± 3.3	94.3 ± 5.7	79.2 ± 4.8	0.49	1.63
E1	123 ± 14.6	104 ± 7.4	104 ± 12.3	86.9 ± 5.2	0.20	0.68	95.4 ± 1.6	88.9 ± 6.2	89.1 ± 15.4	85.1 ± 4.4	0.05	0.17
DES	42.8 ± 3.9	44.0 ± 2.9	54.4 ± 8.8	41.9 ± 3.1	0.63	2.09	59.1 ± 4.3	44.2 ± 4.3	60.7 ± 3.4	51.8 ± 2.4	0.16	0.52

[a] ADD, androsta-1,4-diene-3,17-dione; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; 5α-DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; EE2, 17α-ethynyl estradiol; E1, estrone; DES, diethylstilbestrol.
[b] Mean (%) ± standard deviation (%) (n = 3, replicate samples at the same time).
[c] LOD, method limit of detection; LOQ, method limit of quantitation.

Table 5
Recoveries and detection limits of steroids in surface water samples by RRLC–MS/MS.

Compound[a]	Surface water[b]			LOD[c] (ng/L)	LOQ[c] (ng/L)
	5 ng/L	100 ng/L	Matrix%		
Prednisone	106 ± 6.1	100 ± 4.2	104 ± 4.5	0.05	0.18
Cortisone	111 ± 0.6	90.6 ± 1.3	87.4 ± 4.3	0.07	0.24
Cortisol	107 ± 1.2	97.2 ± 3.0	97.8 ± 2.1	0.06	0.20
Prednisolone	130 ± 1.2	112 ± 6.6	111 ± 5.3	0.03	0.09
Dexamethasone	130 ± 4.4	116 ± 2.6	114 ± 2.6	0.04	0.13
ADD	97.6 ± 5.9	111 ± 4.8	105 ± 1.2	0.05	0.16
17α-Trenbolone	73.4 ± 2.9	93.3 ± 4.8	103 ± 2.0	0.11	0.36
17β-Trenbolone	68.2 ± 4.0	94.2 ± 3.9	113 ± 1.3	0.20	0.68
17β-Boldenone	113 ± 1.3	107 ± 2.8	106 ± 0.8	0.06	0.19
4-Androstene-3,17-dione	107 ± 1.9	110 ± 2.9	107 ± 2.1	0.05	0.16
19-Nortestoserone	88.6 ± 2.5	101 ± 3.2	101 ± 2.3	0.10	0.33
19-Norethindrone	53.8 ± 3.4	93.3 ± 1.5	97.3 ± 0.6	0.02	0.08
17α-Boldenone	88.8 ± 2.0	111 ± 3.3	107 ± 1.1	0.05	0.18
Ethynyl testosterone	89.9 ± 4.1	99.5 ± 2.7	92.4 ± 1.1	0.04	0.13
Testosterone	106 ± 1.0	103 ± 1.8	99.1 ± 0.3	0.05	0.18
Norgestrel	87.5 ± 3.9	96.8 ± 2.8	97.9 ± 1.4	0.04	0.12
Epi-androsterone	113 ± 4.9	119 ± 11.4	101 ± 3.1	0.04	0.14
4-OHA	75.2 ± 12.4	96.7 ± 3.1	75.7 ± 2.0	0.08	0.25
Methyltestosterone	133 ± 0.7	99.2 ± 1.8	71.7 ± 1.2	0.01	0.04
5α-DHT	164 ± 10.4	143 ± 3.4	82.3 ± 11.9	0.04	0.13
Medroxyprogesterone	99.6 ± 1.5	110 ± 2.1	118 ± 0.8	0.04	0.13
Androsterone	106 ± 4.2	113 ± 11.0	111 ± 3.0	0.04	0.14
Progesterone	92.4 ± 6.4	102 ± 1.0	98.0 ± 0.8	0.05	0.17
Stanozolol	91.5 ± 2.5	102 ± 1.0	98.2 ± 0.5	0.01	0.03
E2	104 ± 0.9	107 ± 7.6	89.5 ± 6.6	0.24	0.80
EE2	72.9 ± 8.4	96.3 ± 3.3	97.0 ± 5.5	0.19	0.64
E1	88.2 ± 1.6	113 ± 3.8	101 ± 0.5	0.09	0.30
DES	48.1 ± 1.1	96.2 ± 6.2	80.4 ± 1.8	0.14	0.46

[a] ADD, androsta-1,4-diene-3,17-dione; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; 5α-DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; EE2, 17α-ethynyl estradiol; E1, estrone; DES, diethylstilbestrol.
[b] Mean (%) ± standard deviation (%) (n = 3, replicate samples at the same time).
[c] LOD, limit of method detection; LOQ, limit of method quantitation.

Table 6
Recoveries and detection limits of steroids in sludge samples by RRLC–MS/MS.

Compound[a]	Spiked concentration[b]			Matrix%	LOD[c] (ng/g)	LOQ[c] (ng/g)
	40 ng/g	100 ng/g	200 ng/g			
Prednisone	135 ± 19.7[c]	107 ± 6.6	102 ± 0.2	99.8 ± 2.7	0.84	2.79
Cortisone	85.3 ± 3.8	70.3 ± 4.7	68.7 ± 0.3	67.9 ± 1.9	0.58	1.95
Cortisol	214 ± 2.8	100 ± 9.4	101 ± 5.1	81.7 ± 0.6	1.66	5.54
Prednisolone	83.6 ± 8.3	65.6 ± 3.7	57.9 ± 0.1	50.0 ± 1.2	1.48	4.93
Dexamethasone	136 ± 10.9	116 ± 4.0	122 ± 5.7	102 ± 2.5	2.06	6.86
ADD	115 ± 7.2	112 ± 14.6	115 ± 8.3	104 ± 5.8	0.30	0.99
17α-Trenbolone	107 ± 1.4	100 ± 5.4	104 ± 6.7	99.0 ± 5.9	0.62	2.07
17β-Trenbolone	95.6 ± 7.8	91.6 ± 3.6	94.1 ± 7.6	89.4 ± 3.1	0.64	2.14
17β-Boldenone	104 ± 7.6	101 ± 6.2	105 ± 4.1	90.1 ± 0.7	0.63	2.10
4-Androstene-3,17-dione	85.8 ± 6.1	87.2 ± 6.5	93.7 ± 3.3	92.0 ± 3.7	0.47	1.56
19-Nortestoserone	75.9 ± 7.9	76.5 ± 4.3	85.2 ± 2.3	85.1 ± 0.9	0.58	1.95
19-Norethindrone	88.6 ± 8.0	105 ± 6.6	118 ± 10.4	111 ± 4.5	1.92	6.39
17α-Boldenone	136 ± 3.8	121 ± 9.7	123 ± 7.4	107 ± 10.8	0.41	1.36
Ethynyl testosterone	117 ± 10.1	138 ± 7.6	146 ± 8.8	123 ± 6.2	0.54	1.81
Testosterone	102 ± 4.5	98.9 ± 1.5	101 ± 1.1	98.6 ± 1.0	0.46	1.52
Norgestrel	105 ± 14.2	109 ± 2.9	115 ± 6.6	105 ± 7.4	0.90	2.99
Epi-androsterone	130 ± 39.3	62.6 ± 15.0	43.0 ± 9.4	106 ± 3.5	0.83	2.76
4-OHA	96.0 ± 7.6	94.0 ± 7.3	84.1 ± 2.7	75.4 ± 2.6	0.25	0.83
Methyltestosterone	87.5 ± 5.2	77.2 ± 3.1	76.2 ± 2.0	79.3 ± 4.9	0.37	1.24
5α-DHT	127 ± 14.7	122 ± 10.5	86.1 ± 3.0	95.3 ± 1.8	0.70	2.32
Medroxyprogesterone	136 ± 4.2	137 ± 4.2	140 ± 1.5	122 ± 1.5	0.38	1.28
Androsterone	100 ± 12.6	82.6 ± 5.8	68.1 ± 2.6	69.8 ± 3.5	0.70	2.32
Progesterone	119 ± 5.1	113 ± 2.4	116 ± 3.0	106 ± 0.6	0.42	1.39
Stanozolol	104 ± 3.5	100 ± 2.1	101 ± 3.2	96.5 ± 0.2	0.08	0.27
E2	103 ± 7.9	94.9 ± 2.3	101 ± 0.6	106 ± 13.1	0.98	3.26
EE2	77.3 ± 7.4	69.1 ± 7.9	70.0 ± 2.5	63.0 ± 5.4	0.80	2.66
E1	110 ± 7.8	103 ± 4.0	104 ± 5.9	105 ± 2.5	0.10	0.34
DES	77.3 ± 12.9	76.3 ± 5.7	74.0 ± 7.5	100 ± 8.0	0.59	1.96

[a] ADD, androsta-1,4-diene-3,17-dione; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; 5α-DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; EE2, 17α-ethynyl estradiol; E1, estrone; DES, diethylstilbestrol.
[b] Mean (%) ± standard deviation (%) (n = 3, replicate samples at the same time).
[c] LOD, method limit of detection; LOQ, method limit of quantitation.

Table 7
Concentrations of steroids in wastewater and sludge samples from Huiyang WWTP.

Compound[a]	Wastewater (ng/L)						Sludge (ng/g)			
	Influent	Grit chamber	Anoxic	Anaerobic	Aerobic	Effluent	Anoxic	Anaerobic	Aerobic	Dewatered
Glucocorticoids										
Prednisone	8.5 ± 2.9[b]	4.4 ± 1.1	ND[c]	ND	ND	ND	ND	ND	ND	ND
Cortisone	45.8 ± 7.3	45.9 ± 3.5	2.4 ± 0.3	ND	ND	ND	ND	ND	ND	ND
Cortisol	28.8 ± 0.6	27.3 ± 1.3	2.5 ± 0.2	ND	ND	ND	ND	ND	ND	ND
Dexamethasone	22.6 ± 4.0	13.6 ± 0.8	3.4 ± 1.3	2.3 ± 1.7	ND	ND	ND	ND	ND	ND
Androgens										
ADD	232 ± 5.0	308 ± 12.4	4.4 ± 0.0	4.5 ± 0.2	3.3 ± 0.1	3.2 ± 0.0	24.6 ± 0.5	17.7 ± 1.2	11.4 ± 0.2	37.8 ± 0.4
17α-Trenbolone	2.4 ± 0.8	<LOQ[d]	ND	ND	ND	ND	<LOQ	<LOQ	5.2 ± 0.8	ND
17β-Trenbolone	2.7 ± 2.4	2.0 ± 1.4	ND	ND	ND	ND	ND	ND	ND	ND
17β-Boldenone	37.7 ± 2.4	50.7 ± 2.7	<LOQ	ND	ND	ND	ND	ND	ND	ND
4-Androstene-3,17-dione	55.4 ± 0.8	74.7 ± 2.5	5.5 ± 0.2	5.8 ± 0.2	3.4 ± 0.3	3.4 ± 0.2	18.4 ± 0.6	17.0 ± 1.0	13.0 ± 0.1	12.2 ± 0.2
19-Nortestoserone	ND	ND	ND	ND	ND	ND	ND	ND	ND	7.2 ± 2.6
17α-Boldenone	7.7 ± 4.0	4.9 ± 0.5	3.8 ± 0.3	3.3 ± 0.2	4.9 ± 0.7	13.9 ± 2.7	ND	ND	ND	ND
Testosterone	13.3 ± 0.2	13.2 ± 0.7	<LOQ	<LOQ	<LOQ	<LOQ	2.8 ± 0.1	3.3 ± 0.2	3.7 ± 0.1	<LOQ
Epi-androsterone	620 ± 45.3	479 ± 35.0	18.2 ± 2.3	ND	9.3 ± 8.1	ND	141 ± 17.5	289 ± 7.8	206 ± 6.2	372 ± 38.0
4-OHA	80.9 ± 5.8	89.1 ± 7.8	16.1 ± 4.4	8.2 ± 1.5	16.2 ± 1.4	16.8 ± 3.7	ND	ND	ND	ND
Methyltestosterone	ND	ND	ND	1.0 ± 0.0	1.4 ± 0.3	1.5 ± 0.2	ND	ND	ND	ND
5α-DHT	621 ± 54.0	416 ± 0.5	ND	66 ± 6.4	43.6 ± 13.6	77.3 ± 33.6	ND	ND	ND	ND
Androsterone	305 ± 13.6	240 ± 7.0	ND	ND	ND	ND	ND	ND	ND	ND
Stanozolol	ND	ND	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	ND
Progestagens										
Norgestrel	59.0 ± 28.3	47.0 ± 6.9	19.9 ± 0.8	16.2 ± 1.5	6.9 ± 0.0	9.2 ± 1.0	ND	ND	ND	ND
Ethynyl testosterone	9.4 ± 16.3	ND	ND	ND	ND	ND	ND	ND	ND	ND
Progesterone	6.1 ± 0.3	6.9 ± 0.3	2.9 ± 0.2	0.9 ± 0.0	ND	ND	61.1 ± 0.8	9.4 ± 1.6	7.3 ± 0.4	24.6 ± 3.0
Estrogens										
E2	ND	ND	ND	ND	ND	ND	6.9 ± 0.3	6.4 ± 0.4	3.9 ± 0.6	ND
E1	40.6 ± 10.6	29.9 ± 5.3	30.4 ± 0.8	19.0 ± 1.2	9.8 ± 0.8	8.5 ± 0.4	2.9 ± 0.8	4.3 ± 1.6	2.7 ± 0.7	4.8 ± 0.2

[a] ADD, androsta-1,4-diene-3,17-dione; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; 5α-DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; E1, estrone.
[b] Mean ± standard deviation (n = 3, replicate samples at the same time).
[c] Not detected.
[d] Below the limit of quantitation.

caused by the elevating chromatographic baseline due to some interferences existing in the sludge samples.

Both intra- and inter-day precisions of the RRLC–MS/MS instrument were examined. For the intra-day precision, a standard solution (10 μg/L of each compound) was injected successively seven times. The RSD was in the 0.6–11.6% range for all compounds. For the inter-day experiment, five of the standard solutions (10 μg/L of each compound) were performed on five different

Table 8
Concentrations of steroids in wastewater and sludge samples from Meihu WWTP.

Compound[a]	Wastewater (ng/L)						Sludge (ng/g)			
	Influent	Grit chamber	Anoxic	Anaerobic	Aerobic	Effluent	Anoxic	Anaerobic	Aerobic	Dewatered
Glucocorticoids										
Cortisone	14.5 ± 0.6[b]	13.7 ± 0.4	ND[c]	1.0 ± 0.5	ND	ND	ND	ND	ND	ND
Cortisol	12.7 ± 0.5	9.8 ± 1.1	<LOQ[d]	ND	ND	ND	ND	ND	ND	ND
Dexamethasone	3.8 ± 1.9	2.4 ± 1.2	ND	ND	ND	ND	ND	ND	ND	ND
Androgens										
ADD	170 ± 2.7	141 ± 4.2	6.4 ± 0.6	4.7 ± 0.1	5.0 ± 0.1	2.0 ± 0.1	12.9 ± 0.3	14.9 ± 0.7	12.3 ± 0.4	12.4 ± 0.2
17α-Trenbolone	2.7 ± 1.6	3.3 ± 1.2	ND	ND	ND	ND	5.4 ± 0.3	4.1 ± 0.9	5.7 ± 1.8	10.4 ± 0.2
17β-Trenbolone	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND	4.6 ± 1.8
17β-Boldenone	17.3 ± 1.4	15 ± 0.6	ND	ND	ND	ND	ND	ND	ND	ND
4-Androstene-3,17-dione	45.8 ± 1.3	36.7 ± 1.3	6.0 ± 0.2	3.2 ± 0.2	3.8 ± 0.1	2.1 ± 0.0	13.0 ± 0.2	16.2 ± 0.2	12.6 ± 0.1	9.0 ± 0.2
17α-Boldenone	6.3 ± 0.6	ND	3.3 ± 0.3	2.9 ± 0.1	ND	10.9 ± 14.5	ND	ND	ND	ND
Testosterone	5.4 ± 0.4	5.2 ± 0.4	<LOQ	<LOQ	<LOQ	<LOQ	3.5 ± 0.1	3.2 ± 0.2	3.4 ± 0.1	ND
Epi-androsterone	280 ± 16.1	321 ± 19.4	11.5 ± 2.7	ND	ND	ND	216 ± 7.0	265 ± 13.7	209 ± 17.1	338 ± 40.6
4-OHA	102 ± 3.5	67.5 ± 4.7	32.4 ± 7.1	10.5 ± 2.4	5.1 ± 1.5	17.3 ± 6.6	ND	ND	ND	ND
Methyltestosterone	1.8 ± 0.1	ND	1.8 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.3 ± 0.4	ND	ND	ND	ND
5α-DHT	326 ± 6.2	271 ± 8.9	ND	ND	ND	ND	ND	ND	ND	94.0 ± 2.8
Androsterone	125 ± 10.4	154 ± 3.7	ND	ND	ND	ND	ND	ND	ND	ND
Stanozolol	ND	ND	ND	ND	ND	ND	<LOQ	<LOQ	<LOQ	ND
Progestagens										
Norgestrel	28.7 ± 3.7	21.6 ± 2.0	20.1 ± 2.0	18.6 ± 1.4	11.5 ± 0.9	6.7 ± 1.2	ND	ND	ND	ND
Progesterone	5.4 ± 0.6	3.1 ± 0.0	3.8 ± 0.2	ND	0.3 ± 0.0	ND	6.4 ± 0.2	11.8 ± 0.2	6.2 ± 0.2	6.0 ± 0.2
Estrogens										
E2	ND	ND	ND	ND	ND	ND	10.2 ± 1.3	6.0 ± 0.7	4.6 ± 1.1	ND
E1	21.7 ± 0.7	23.8 ± 1.7	44.5 ± 1.2	41.0 ± 1.6	9.7 ± 1.3	3.1 ± 0.5	3.3 ± 1.1	2.2 ± 0.8	1.6 ± 0.4	5.4 ± 0.2

[a] ADD, androsta-1,4-diene-3,17-dione; 4-OHA, 4-hydroxy-androst-4-ene-17-dione; 5α-DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; E1, estrone.
[b] Mean ± standard deviation (n = 3, replicate samples at the same time).
[c] Not detected.
[d] Below the limit of quantitation.

Table 9
Concentrations of target compounds in surface water samples of Danshui River.

Location	Compound[a] (ng/L)									
	Cortisone	ADD	17β-Boldenone	4-Androstene-3,17-dione	Testosterone	Norgestrel	Epi-androsterone	5α-DHT	Progesterone	E1
Upstream	0.6 ± 0.1[b]	8.2 ± 0.6	0.4 ± 0.1	8.1 ± 0.7	ND[c]	3.7 ± 0.3	ND	38.6 ± 12.6	0.5 ± 0.1	6.0 ± 0.6
Downstream	1.9 ± 0.0	17.9 ± 0.2	1.5 ± 0.2	8.6 ± 0.6	1.2 ± 0.1	22.2 ± 0.3	27.6 ± 6.3	55.3 ± 33.3	2.5 ± 0.1	13.3 ± 1.3

[a] ADD, androsta-1,4-diene-3,17-dione; 5α-DHT, 5α-dihydrotestosterone; E1, estrone.
[b] Mean ± standard deviation (n = 3, replicate samples at the same time).
[c] Not detected.

days over one month interval. In this case the RSD was less than 14.1%.

3.7. Application to real samples

Two surface water, twelve wastewater and eight sludge samples were analyzed by the developed analytical method. The mean concentrations of the detected analytes were reported in Tables 7–9. ADD, 4-androstene-3,17-dione, T, norgestrel, 4-OHA and E1 could be detected in every wastewater sample. Ten analytes (cortisone, ADD, 17β-boldenone, 4-androstene-3,17-dione, T, norgestrel, EARD, 5α-DHT, P, E1) were detected in surface water samples, twenty of 28 targets (except prednisolone, 19-nortestoserone, 19-norethindrone, S, MP, E2, EE2, DES) were detected in the influent samples (1.8 (MT)–621 ng/L (5α-DHT)), while 9 (ADD, 4-androstene-3,17-dione, 17α-boldenone, T, norgestrel, 4-OHA, MT, 5α-DHT, E1) were detected in the effluent samples (1.2 (MT)–77.3 ng/L (5α-DHT)). The concentrations of the detected compounds in the downstream of Danshui River were higher than those in the upstream and even effluent from Huiyang WWTP. It is mainly due to discharge of some other sources, such as untreated sewage in the upstream of the river.

Significant removal in the two WWTPs has been observed for most of the detected steroids. However, the concentrations for MT were relatively stable in different treatment stages of Meihu WWTP with concentrations varying from 1.8 ng/L in influent to 1.3 ng/L in effluent; but its concentrations increased from not detected in influent to 1.5 ng/L in effluent in Huiyang WWTP. It should be noted that the concentrations of 17α-boldenone increased in the effluents compared with those in the influents. This may be due to the fact that some kinds of biological conversion or the deconjugation of glucuronide and sulfate conjugated steroids occurred in the wastewater treatment processes [28,29], and it requires further investigation.

No glucocorticoid steroids were detected in the sludge samples. ADD, 4-androstene-3,17-dione, T, EADR, P, and E1 could be detected in each sludge sample of different stages at similar concentration levels. The concentrations of EADR in the sludge samples were rather high ranging between 141 and 372 ng/g.

Comparing the detected steroids in the wastewater with those in the sludge samples, we found that ADD, 4-androstene-3,17-dione, T, and E1 could be detected in all samples (wastewater and sludge), while norgestrel, 4-OHA, MT, prednisone, cortisone, cortisol, dexamethasone, ADR, 17α-boldenone, and 17β-boldenone could be detected only in wastewater. It suggested that these steroids were more inclined to partition to water phase rather than in the sludge phase, while E2 and S could only be detected in the sludge samples of these two WWTPs. Many factors such as rapid biodegradation and polarity could be responsible for the different detection in the two phases. For example, E2 has been reported in wastewater [28,30,31], but not detected in this study indicating conversion to E1 or rapid biodegradation in the sewage systems especially in such as a warm region like Guangdong.

4. Conclusion

A sensitive and reliable analytical method was developed to simultaneously determine the concentrations of four classes of steroids in surface water, wastewater and sludge samples. And this is the first time to use ultrasonic extraction simultaneously to extract these 28 steroids in sludge samples. The optimized extraction method for sludge samples used ethyl acetate as the extraction solvent, and showed the best extraction efficiencies for the 28 target compounds. Simple and effective cleanup of the different water or sludge extracts was performed by self-made silica gel cartridges with ethyl acetate mixed with methanol (90:10, v/v) as the elution solvent. Purified extracts were analyzed by RRLC–MS/MS that operated in both negative (Group I) and positive (Group II) mode. For most analytes, this developed method shows good recoveries, no significant matrix effects, and is suitable for analyzing the target compounds in different water or sludge samples with low LODs at sub-ng/L or sub-ng/g, respectively.

This sensitive method was successfully applied to the analysis of 12 wastewater and 8 sludge samples from two WWTPs. The concentration of detected steroids in surface water, wastewater and sludge samples varied greatly, from 0.4 to 55.3 ng/L, 0.3 to 621 ng/L, 1.6 to 372 ng/g, respectively. The most frequent compounds were 4-androstene-3,17-dione, T, ADD, and E1. No glucocorticoid was detected in all sludge and effluent samples.

Acknowledgements

The authors thank for the financial support from National Natural Science Foundation of China (NSFC 20977092, 40688001, 40821003 and 40771180), Guangdong Provincial Natural Science Foundation (8251064004000001) and the Earmarked Fund from the State Key Laboratory of Organic Geochemistry (sklog2009A02). This is Contribution No. 1277 from GIG CAS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.01.014.

References

[1] L.J. Mills, C. Chichester, Sci. Total Environ. 343 (2005) 1.
[2] G.M. Solomon, T. Schettler, Can. Med. Assoc. J. 163 (2000) 1471.
[3] R. Jenkins, R.A. Angus, H. McNatt, W.M. Howell, J.A. Kemppainen, M. Kirk, E.M. Wilson, Environ. Toxicol. Chem. 20 (2001) 1325.
[4] E.F. Orlando, A.S. Kolok, G.A. Binzcik, J.L. Gates, M.K. Horton, C.S. Lambright, L.E. Gray, A.M. Soto, L.J. Guillette, Environ. Health Perspect. 112 (2004) 353.
[5] G. Streck, Trends Anal. Chem. 28 (2009) 635.
[6] J.B. Quintana, J. Carpinteiro, I. Rodriguez, R.A. Lorenzo, A.M. Carro, R. Cela, J. Chromatogr. A 1024 (2004) 177.
[7] W.H. Ding, C.C. Chiang, Rapid Commun. Mass Spectrom. 17 (2003) 56.
[8] A. Shareef, M.J. Angove, J.D. Wells, J. Chromatogr. A 1108 (2006) 121.
[9] K. Zhang, Y.G. Zuo, Anal. Chim. Acta 554 (2005) 190.
[10] M.J. Wheeler, Y.B. Zhong, A.T. Kicman, S.B. Coutts, J. Endocrinol. 159 (1998) R5.
[11] J.A. Bowden, D.M. Colosi, D.C. Mora-Montero, T.J. Garrett, R.A. Yost, J. Chromatogr. B 877 (2009) 3237.

[12] M.S. Diaz-Cruz, M.J.L. de Alda, R. Lopez, D. Barcelo, J. Mass Spectrom. 38 (2003) 917.
[13] P.E. Joos, M. Van Ryckeghem, Anal. Chem. 71 (1999) 4701.
[14] H. Chang, Y. Wan, J.Y. Hu, Environ. Sci. Technol. 43 (2009) 7691.
[15] H. Chang, J.Y. Hu, B. Shao, Environ. Sci. Technol. 41 (2007) 3462.
[16] H. Chang, S.M. Wu, J.Y. Hu, M. Asami, S. Kunikane, J. Chromatogr. A 1195 (2008) 44.
[17] F. Chen, G.-G. Ying, J.-F. Yang, J.-L. Zhao, L. Wang, J. Environ. Sci. Health Part B 45 (2010) 682.
[18] J.L. Zhao, G.G. Ying, Y.S. Liu, F. Chen, J.F. Yang, L. Wang, J. Hazard. Mater. 179 (2010) 215.
[19] J. Xu, L.S. Wu, W.P. Chen, A.C. Chang, J. Chromatogr. A 1202 (2008) 189.
[20] R. Liu, J.L. Zhou, A. Wilding, J. Chromatogr. A 1038 (2004) 19.
[21] A. Nieto, F. Borrull, E. Pocurull, R.M. Marce, Environ. Toxicol. Chem. 29 (2010) 1484.
[22] M.Y. Song, Y. Xu, Q.T. Jiang, P.K.S. Lam, D.K. O'Toole, J.P. Giesy, G.B. Jiang, Environ. Int. 32 (2006) 676.
[23] X.Z. Peng, Z.D. Wang, C. Yang, F.R. Chen, B.X. Mai, J. Chromatogr. A 1116 (2006) 51.
[24] P. Labadie, E.M. Hill, J. Chromatogr. A 1141 (2007) 174.
[25] N. Gineys, B. Giroud, E. Vulliet, Anal. Bioanal. Chem. 397 (2010) 2295.
[26] T. Benijts, R. Dams, W. Lambert, A. De Leenheer, J. Chromatogr. A 1029 (2004) 153.
[27] B.J. Vanderford, R.A. Pearson, D.J. Rexing, S.A. Snyder, Anal. Chem. 75 (2003) 6265.
[28] C. Baronti, R. Curini, G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Samperi, Environ. Sci. Technol. 34 (2000) 5059.
[29] R.L. Gomes, M.D. Scrimshaw, J.N. Lester, Environ. Sci. Technol. 43 (2009) 3612.
[30] P. Labadie, H. Budzinski, Environ. Sci. Technol. 39 (2005) 5113.
[31] T.A. Ternes, M. Stumpf, J. Mueller, K. Haberer, R.D. Wilken, M. Servos, Sci. Total Environ. 225 (1999) 81.