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RESEARCH PAPER

Analysis of 21 progestagens in various matrices by ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with diverse sample pretreatment

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Abstract In this study, a highly sensitive and robust method using an ultra-high-performance liquid chromatography-tandem mass spectrometry combined with solid-phase extraction and ultrasonic extraction for pretreatment and silica gel purification steps has been developed for determination of 21 natural and synthetic progestagens in river surface water and sediments, and influents, effluents, and sludge from municipal wastewater treatment plants, and flush water and feces from swine farms. For the various matrices considered, the optimized method showed satisfactory performance with recoveries of 70–129 % (except AD, 5α-DHP, DPT, HPC), the limits of quantification below 2.30 ng/L for liquid samples and 2.59 ng/g for solid samples (except AD), and good linearity and reproducibility. This developed method was successfully applied in the analysis of progestagens in environmental samples from Liuxi Reservoir, Xintang municipal wastewater treatment plant, and Shunfeng swine farm in South China. Six analytes were detected at trace levels in surface water, effluent, and sediment samples. Seven analytes (0.7 (HPA)–35.1 ng/L (DGT)) were found in the influent samples and three analytes (5.6 (DGT)–11.8 ng/g (5α-DHP)) in the dewatered sludge samples. Moreover, 13 analytes were detected in swine farm, with high concentrations ranging from 23.8 ng/L (ET) to 5,024 ng/L (P) in flush water, and from 20.0 ng/g (MPA) to 1952 ng/g (P) in feces.

Keywords Progestagens · Environment matrices · Extraction · Purification · UHPLC-MS/MS

Introduction

In recent years, steroid hormones in the environment have become a public concern due to their potential endocrine disrupting effects [1–3]. Previous environmental studies about steroid hormones mostly focused on estrogens [4–7] and a few studies on other steroids such as progestagens [8–11]. It is now well established that various natural and synthetic progestagens are widely used in humans and animals daily life for many reasons [12, 13], and recent studies have shown the reproductive toxicity of progestagens to aquatic organisms at nanograms per liter levels [14–19]. Therefore, the presence of progestogens in the environment should deserve greater attention, and it is essential to develop sensitive and reliable analytical methods for determination of the broad number of progestagens in various environmental matrices in order to assess their environmental risks.

In previous studies, solid-phase extraction (SPE) is a commonly used extraction technique, with the moderate cost and simple operation, widely used in liquid samples [12, 13, 20–23]. For solid samples, there are various available extraction techniques for steroid hormones, such as ultrasonic extraction (USE) [10, 11, 24], microwave-assisted extraction [25], accelerated solvent extraction (ASE) [26], and Soxhlet extraction [27]. For instrumental analysis, gas

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chromatography-mass spectrometry (GC-MS) has been applied in determination of steroids due to its high separation and good identification capability [20–22]. However, derivatization steps are required before instrumental analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers an alternate choice for determination of progestagens with the advantages of reduced analytical time and no derivatization steps. It has increasingly been applied to analyze progestagens in different environmental matrices due to its simplicity of operation, high selectivity, and excellent sensitivity [10–13, 28]. In our previous study, a method for trace analysis of 28 steroids in surface water, wastewater, and sludge samples by LC-MS-MS was developed, which contained five progestagens [11]. Actually, a large number of progestagens are used as drugs in human and animals; unfortunately, only a limited number of progestagen compounds were included in previously reported methods [11, 13, 21, 28, 29]. It is necessary to develop a specialized method for simultaneous screening of various progestagens in more diverse environmental matrices.

The objective of this study was to develop a sensitive, robust, and reliable analytical method for simultaneous determination of 21 progestagens in environmental samples including surface water, sediment, wastewater, flush water, sludge, and feces samples by using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Pretreatment of samples involved SPE for liquid samples, and ultrasonic extraction and silica gel cleanup for solid samples. Experimental conditions were optimized for these progestagens in terms of recovery and sensitivity. Finally, the developed method was applied to determine the target compounds in real environmental samples including flush water and feces from a swine farm, wastewater, and sludge from a wastewater treatment plant (WWTP), as well as surface water and sediment from the receiving environments.

Materials and methods

Chemicals and materials

High purity authentic standards of 21 progestagens, including anordrin (AD), chlormadinone (CMD), chlormadinone acetate (CMDA), cyproterone acetate (CPRA), dydrogesterone (DGT), 5α-dihydroprogesterone(5α-DHP), drospirenone (DPN), ethynyl testosterone (ET), hydroxy progesterone (HP), 17α-hydroxyprogesterone acetate (17α-HPA), hydroxyprogesterone caproate (HPC), mifepristone (MFST), melengestrol acetate (MGA), megestrol (MGT), medroxyprogesterone (MP), medroxyprogesterone acetate (MPA), norgestrel (N), norethynodrel (NTD), 19-norethindrone (19-NTD), norethisterone acetate (NTRA), progesterone (P),and corresponding internal standards melengestrol acetate-d3 (MGA-d3), mifepristone-d3 (MFST-d3), progesterone-d9 (P-d9), norethindrone-d6 (NTD-d6) were purchased from Meryer Technologies Co.(China), USP, Dr. Ehrenstorfer GmbH (Germany), Steraloids Inc. (USA), Sigma-Aldrich (USA), and TCR (North York, Canada), respectively (Table 1).

All reagents of HPLC-grade (including methanol (MeOH), acetonitrile (ACN), ethyl acetate (EtOAc), hexane (Hex), and dichloromethane (DCM)) were obtained from Merck (Darmstadt, Germany) or CNW Technologies (Dusseldorf, Germany). Formic acid and acetic acid were obtained from Tedia company (Fairfield, OH, USA), and ammonium acetate from Sigma-Aldrich (Saint Louis, MO, USA).

The cartridges used for SPE were Oasis HLB cartridges (*N*-vinylpyrrolidone-m-divinylbenzene copolymer, 500 mg, 6 mL) from Waters (Milford, MA, USA), Super clean ENVI-18 cartridges (500 mg, 6 mL) from Supelco, and Bond Elut cartridges (500 mg, 6 mL) from Agilent (Agilent, USA). Glass fiber filters (GF/F, pore size 0.7 μm) were purchased from Whatman (Maidstone, UK) 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). 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.

Individual stock solutions of all chemicals were prepared at a concentration of 100 mg/L in MeOH. Mixed standard solution and internal standard solution for UHPLC-MS/MS analysis were prepared at 1 mg/L in MeOH. The working standard solutions were prepared weekly. All the standard solutions were stored in amber glass bottles and kept at −18 °C in a freezer before use.

Sample collection

For the method establishment and application tests, different environmental samples were collected. Surface water and sediment samples were from Liuxi Reservoir located in Conghua, north of Guangzhou. Flush water and feces samples were from Shunfeng swine farm in Jiangmen, Guangdong province. Wastewater and dewatered sludge samples were from Xintang WWTP in Guangzhou. Meanwhile, we also collected surface water from a river that receives the discharge of WWTP effluents. Three parallel samples were collected from each sampling location. For liquid samples, about 50 mL of MeOH was added into each bottle (1 L), and then its pH was adjusted to 3 using 4 M H_2SO_4 in the field. For solid samples, approximately 1 g of sodium azide was added to 1 kg

Table 1 Details of the target compounds and their MRM parameters in UHPLC-MS/MS under positive ionization mode

Compound[a]	Supplier	M. W. [b]	CAS	R.T. [c] (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	Fragmentor (eV)	Collision energy (eV)	Corresponding I.S.[a]
Anordrin	Steraloids Inc.	438.3	56470-64-5	15.314	439.3	383.3	105	4	Norethindrone-d6
						365.3	105	4	
Chlormadinone	TRC	362.2	1961-77-9	5.822	363.2	43.1	110	48	Progesterone-d9
						309.2	110	20	
Chlormadinone acetate	Dr. Ehrenstorfer	404.2	302-22-7	6.752	405.2	301.1	120	16	Melengestrol acetate-d3
						43.1	120	56	
Cyproterone acetate	Meryer	416.2	427-51-0	6.146	417.2	43.1	135	64	Progesterone-d9
						147.1	135	20	
Dydrogesterone	USP	312.2	152-62-5	6.745	313.2	43.1	110	56	Progesterone-d9
						77.1	110	92	
5α-Dihydroprogesterone	Sigma	316.2	566-65-4	9.724	317.2	43.1	115	68	Progesterone-d9
						91.1	115	64	
Drospirenone	TRC	366.2	67392-87-4	4.721	367.2	97.1	140	28	Progesterone-d9
						91.1	140	48	
Ethynyl testosterone	Dr. Ehrenstorfer	312.2	434-03-7	5.205	313.2	109.1	140	28	Progesterone-d9
						97.1	140	24	
Hydroxy progesterone	Dr. Ehrenstorfer	330.2	68-96-2	5.285	331.2	109.1	125	28	Progesterone-d9
						97.1	125	28	
17α-Hydroxyprogesterone acetate	Dr. Ehrenstorfer	372.2	302-23-8	5.970	373.2	97.1	125	28	Melengestrol acetate-d3
						109.1	125	28	
Hydroxyprogesterone caproate	Meryer	428.3	630-56-8	12.410	429.3	43.2	145	56	Melengestrol acetate-d3
						97.1	145	40	
Mifepristone	Meryer	429.3	84371-65-3	8.839	430.3	134.1	165	36	Mifepristone-d3
						372.2	165	20	
Mifepristone-d3 (I.S.)	TRC	432.3	–	8.716	433.3	137.1	170	44	–
						375.3	170	20	
Melengestrol acetate	Dr. Ehrenstorfer	396.2	2919-66-6	7.552	397.2	236.2	115	28	Melengestrol acetate-d3
						297.2	115	16	
Melengestrol acetate-d3 (I.S.)	TRC	399.3	–	7.522	400.3	221.1	120	44	–
						279.2	120	16	
Megestrol	Meryer	342.2	3562-63-8	6.080	343.2	187.1	135	20	Progesterone-d9
						43.1	135	76	
Medroxyprogesterone	Dr. Ehrenstorfer	344.2	520-85-4	6.486	345.2	123.1	140	24	Progesterone-d9
						97.1	140	24	
Medroxyprogesterone acetate	Dr. Ehrenstorfer	386.3	71-58-9	7.228	387.3	123.1	135	28	Melengestrol acetate-d3
						97.1	135	36	
Norgestrel	Sigma	312.2	6533-00-2	5.835	313.2	109.1	125	28	Norethindrone-d6
						91.1	125	56	
Norethynodrel	TRC	298.2	68-23-5	4.491	299.2	91.0	80	56	Norethindrone-d6
						281.2	80	12	
19-Norethindrone	TRC	298.2	68-22-4	4.605	299.2	109.1	135	28	Norethindrone-d6
						77.1	135	76	
Norethindrone-d6 (I.S.)	TRC	304.2	–	4.423	305.2	91.1	125	52	–
						114.1	125	24	
Norethisterone acetate	Dr. Ehrenstorfer	340.2	51-98-9	6.991	341.2	91.1	125	56	Melengestrol acetate-d3
						109.1	125	28	
Progesterone	Dr. Ehrenstorfer	314.2	57-83-0	7.909	315.2	97.1	145	24	–

Table 1 (continued)

Compound[a]	Supplier	M. W. [b]	CAS	R.T. [c] (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	Fragmentor (eV)	Collision energy (eV)	Corresponding I.S.[a]
Progesterone-d9 (I.S.)	TRC	323.3	15775-74-3	7.778	324.3	109.1	145	24	Progesterone-d9
						113.1	120	24	
						100.1	120	24	

[a] I.S. internal standard

[b] Molecular weight

[c] Retention time (minutes)

of each sample to suppress microbial activity. Water samples were transported back to the laboratory and stored in the dark at 4 °C, and processed within 48 h. Sediment and dewater sludge samples were lyophilized, homogenized, and packed with clean aluminum foils, and then stored in 4 °C for further use.

Sample extraction and purification

Water sample extraction

Water samples (surface water, influent, effluent, and flush water) were extracted by SPE. Firstly, water samples (1 L surface water and effluent, 500 mL influent, and 200 mL flush water) were filtered through glass fiber filters (Whatman GF/F, 0.7 μm effective pore size, UK) to remove suspended particles. Secondly, exactly 100 ng of the internal standard mixture solution was added to each sample, and for recovery tests, the target compounds were spiked at concentrations of 10, 50, and 100 ng/L in surface water; 20, 50, and 100 ng/L in effluent; 50, 100, and 200 ng/L in influent; and 50, 100, 500, and 5,000 ng/L in flush water. The extraction method for water samples was evaluated by testing three pH values (7.0, 5.0 and 3.0), three SPE cartridges (Oasis HLB, Superclean C18 and Bond Elut), and four elution solvents (EtOAc, DCM, MeOH, and MeOH/DCM (7/5, *v/v*)).

The optimized SPE method is as follows. The SPE cartridge (Oasis HLB, 6 mL, 500 mg) was preconditioned consecutively with 10 mL of MeOH and 10 mL of Milli-Q water. The filtered water samples were loaded onto the SPE cartridges at a flow rate of 5–10 mL/min. Then each sample bottle was rinsed twice with two aliquots of 50 mL of 5 % (*v/v*) MeOH in Milli-Q water, which passed through the cartridge after sample loading. Then the cartridges were dried under the vacuum for 2–3 h, and the target compounds were eluted from each cartridge with 3×4 mL EtOAc. The eluents were dried under a gentle nitrogen stream, then re-dissolved with 1 mL of MeOH and filtered through a 0.22 μm membrane filter (Anple, Shanghai, China) into a 2-mL amber glass vial (Agilent, USA) prior to UHPLC-MS/MS analysis.

Solid sample extraction

Extraction method for solid samples (sediment, sludge and feces) was improved by testing both of USE and ASE, and different extraction solvents (EtOAc, MeOH, EtOAc/MeOH (8/2, 5/5, and 2/8, *v/v*)). Firstly, a lyophilized solid sample (2.0 g of sediment sample, 0.5 g of sludge, and feces samples) was weighed into a 30-mL glass centrifuge tube or an extraction tube. Secondly, exactly 100 ng of the internal standard mixture was added to each sample for recovery tests, and the target compounds were spiked at concentrations of 20, 50, and 100 ng/g in sediment; 50, 100, and 200 ng/g in sludge; and 50, 200, and 2,000 ng/g in feces. The USE tubes were thoroughly mixed, while the ASE tubes were gently mixed. Then all tubes were placed into a fume hood for approximately 3–4 h with foil loosely caped to volatilize the solvent and kept in 4 °C overnight. The optimized ASE conditions were: extraction solvent, MeOH; temperature, 100 °C; flush solvent, 60 %; static time, 5 min; and number of cycles, 3.

The final optimized method for simultaneous extraction of 21 progestagens in solid samples is as follows. USE was selected for the extraction of solid samples. Some 10 mL of EtOAc/MeOH (8/2, *v/v*) was added to a sample tube; the solution was mixed by a vortex mixer about 30 s, extracted in an ultrasonic bath for 15 min, and centrifuged at 1,370×*g* for 10 min. The clear supernatant from each tube was pipetted into a 100-mL pear-shaped flask. The extraction procedure was repeated thrice, and the supernatants from the three extractions were combined, evaporated at 45 °C by a rotary evaporator, then re-dissolved with 1 mL of MeOH, and filtered through a 0.22 μm membrane filter (Anple, Shanghai, China) into a 2-mL amber glass vial (Agilent, USA) prior to further purification.

Purification

In order to reduce the matrix interferences, a further purification step was applied for the extracts of solid samples. Purification was tested by using HLB SPE cartridges or self-made silica gel cartridges. For the cleanup with HLB SPE cartridges,

the combined supernatants were diluted with Milli-Q water (270 mL) to a volume of 300 mL in order to make MeOH content lower than 10 %. The diluted extract was purified by an Oasis HLB cartridge (200 mg, 6 mL) with the elution and reconstitution conditions being the same as the SPE procedure for liquid samples as described in the section on "Water sample extraction". For the clean-up with self-made silica gel cartridges, the purification step was developed by testing different elution solvents (Hex, DCM, EtOAc, and EtOAc/MeOH (9/1, 8/2, 7/3, 6/4, and 5/5, *v/v*)). The optimized purification procedure is as follows. First of all, the empty glass cartridge (18 cm×1 cm i.d.) was filled with glass wool (CNW), 1.0 g silica gel, which had been extracted by DCM for about 48 h, and 0.5 cm of anhydrous sodium sulfate, successively. Each USE methanolic extract (200 μL) was loaded to a silica cartridge, which was preconditioned with 5 mL of MeOH, 5 mL of EtOAc/MeOH (9:1, *v/v*), and 5 mL of Hex. After the cartridge was rinsed with 6 mL of Hex, the targets were eluted with 6 mL of EtOAc/MeOH (9:1, *v/v*). The eluate was then dried and reconstituted in 200 μL in the buffer MeOH/ Milli-Q water–5 mM ammonium acetate–0.05 % formic acid (70/30, *v/v*) before analysis.

Instrumental analysis

The target compounds were analyzed by an Agilent 1200 series ultra-high performance liquid chromatography (Agilent, USA) coupled to an Agilent 6460 triple quadrupole mass spectrometry (UHPLC-MS/MS). The instrumental method was optimized by testing different ionization sources (electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI)), different ionization modes (positive and negative), and different mobile phases (MeOH, ACN, Milli-Q water, Milli-Q water containing 0.01 % acetic acid (*v/v*), Milli-Q water containing 5 mM ammonium acetate, Milli-Q water containing 5 mM ammonium acetate, and 0.05 % formic acid (*v/v*)).

The final optimized method for instrumental analysis of 21 progestagens in environmental samples is as follows. The target compounds were separated on a Zorbax SB-C18 column (100×3 mm, 1.8 μm particle size) with its corresponding pre-column filter (2.1 mm, 0.2 μm) (Agilent). The column oven temperature was maintained at 40 °C, and the injection volume was 5.0 μL. The mobile phase consisted of (A) Milli-Q water containing 5 mM ammonium acetate and 0.05 % formic acid (*v/v*) and (B) MeOH. The gradient program of the mobile phase was 70 % B at 0 min, increased to 90 % B at 13 min, and decreased back to 70 % B at 17 min, at a flow rate of 0.35 mL/min. A post-run time was set at 5.0 min for column equilibration prior to next injection. The mass spectrometry was operated with ESI in positive ionization mode. The MS operating parameters, including fragmentor voltage, collision energy (CE), precursor ion, and product ions for each compound, were optimized by Optimizer (Agilent, USA) to maximize the best signal response and increase detection sensitivity (Table 1). 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. The extracted ion chromatograms (EIC) of the quantitative ions for the target compounds in a 50 μg/L standard solution are shown in Fig. 1. The MS operating conditions were: gas temperature, 350 °C; gas flow, 8 mL/min; nebulizer pressure, 50 psi; sheath gas flow, 12 L/min; sheath gas temperature, 350 °C; nozzle voltage, 0 V; and capillary voltage, 3,500 V.

Quantification and method validation

Data acquisition was performed by Agilent Mass Hunter B 02.01 software, while identification of the target compounds was based on their retention times (within 2 %) and the ratios of the two selected precursor–product ion transitions (within 20 %) in comparison with the corresponding standards. In order to compensate for potential experimental errors and matrix effects, an internal standard method was applied in the quantitative analysis of the target compounds. The mixed isotope-labeled internal standard solution was added before sample extraction. Recovery tests were completed through spiking the mixed standard solutions with different concentrations to surface water, influent, effluent, flush water, sediments, sludge, and feces samples to evaluate the performance of the analytical method. At the same time, the spiking blank was processed for each matrix for determination of the background concentrations of the target compounds, and then the recovery for each compound was calculated according to the formula: $\text{Recovery}=(C_{\text{spiked}}-C_{\text{blank}})/C_{\text{standard}}\times 100\ \%$. The recovery between 70 % and 120 % was regarded as satisfactory. Matrix effect for each compound was evaluated by comparing the matrix extracts spiked with the standard solution to the standards in mobile phase (detailed calculation method is given in the Electronic supplementary material (ESM) p. 15). Method limit of detection (LOD) and limit of quantification (LOQ) of each compound were obtained based on the signal-to-noise ratio (S/N) near the target peak. LOD is defined as three times of S/N under the lowest spiked concentration of different environmental samples, while LOQ is ten times of S/N. Laboratory blanks, reagent blanks, and quality control standard solution (50 μg/L each compound) were also performed with the samples during the analysis of each batch to assess potential background values and instrument performance.

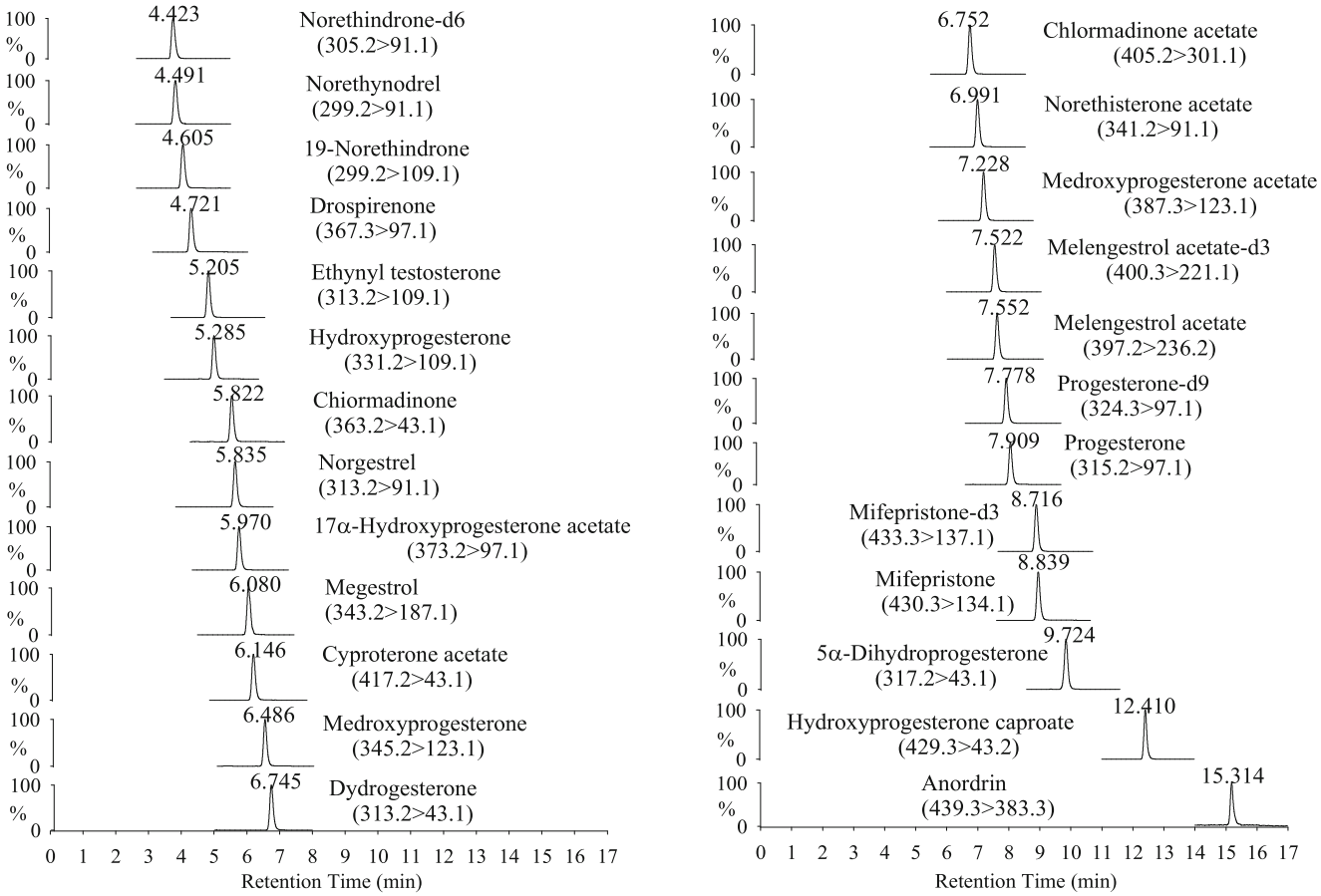

Fig. 1 UHPLC-MS/MS extracted ion chromatograms (EIC) of the quantitative ions for target compounds and corresponding internal standards in 100 μg/L standard solution

Results and discussion

Optimization of water sample extraction

SPE was used to extract the target compounds in water samples. Different SPE cartridges, pH values, and elution solvents were tested during the optimization. All the experiments were performed by spiking standards (100 ng/L each) into 1 L Liuxi Reservoir water.

The recoveries of the 21 target compounds with three different cartridges (Oasis HLB, Superclean C_{18} and Bond Elut) are displayed in Fig. S1 (see ESM). The results showed that most target compounds exhibited good recoveries between 70 % and 120 %. For AD, the Bond Elut cartridges produced the best recoveries (nearly 70 %), followed by ENVI-18 cartridges (near 50 %) and Oasis HLB cartridges (<50 %). For MFST, Oasis HLB cartridges gave the best recoveries (approximately 100 %), but the other two cartridges did not retain the target compound. Oasis HLB cartridges contain the hydrophilic–lipophilic balanced reversed-phase sorbent which shows high retention, good reproducibility, and excellent recovery for the target compounds. Thus, Oasis HLB cartridges were selected for the water sample extraction after taking consideration of all target compounds.

Sample pH value can affect chemical speciation or physicochemical properties of the target compounds; three different pH values (3.0, 5.0 and 7.0) were tested, and the results are shown in Fig. S2 (see ESM). At pH 3.0, the recoveries of all target compounds fell within the range of 70–120 % except for AD having its recoveries of less than approximately 50 %. Figure S2 in the ESM also shows that the recoveries for most of the target compounds at pH 5.0 and 7.0 were higher than those at pH 3.0. Some compounds such as CMD, DGT, and MP had their recoveries more than 120 % at pH 7.0, while MFST had its recoveries less than 50 % at pH 5.0. Moreover, the standard deviations (SD) of the recoveries for all target compounds at pH 3.0 were generally smaller than those at pH 5.0 and 7.0. Adjusting water samples to pH 3.0 in the field can also inhibit bacterial growth and assist sample preservation. Thus, the best pH choice for water sample extraction and preservation was pH 3.0.

Four different elution solvents (EtOAc, MeOH, DCM, and MeOH/DCM (7:5, *v/v*)) were tested in the method development, and the results are given in Fig. S3 (see ESM). It shows that all four elution solvents produced similar recoveries for

the target compounds which fell within the range of 70–120 % except for AD with its recoveries of approximately 50 %. Considering the moderate polarities of the target compounds, EtOAc was regarded as the best choice, since it can minimize the effects by the matrix interferences, and it is also a less toxic solvent than the rest.

Optimization of solid sample extraction

Extraction solvent is a key factor for both USE and ASE methods. According to the properties of the target compounds, five extraction solvents (EtOAc, EtOAc/MeOH (8/2, *v/v*), EtOAc/MeOH (5/5, *v/v*), EtOAc/MeOH (2/8, *v/v*), and MeOH) were tested with sludge samples. The results are given in Figs. 2 and 3. It can be seen that most of the target compounds had good recoveries between 70 % and 120 %. With USE, all five extraction solvents gave the recoveries ranging between 70 % and 120 %, except for AD, with its recoveries of more than 120 %. Among the five solvents, EtOAc/MeOH (8/2, *v/v*) produced the best recovery results (70–120 %) with USE (Fig. 2). With ASE, all extraction solvents produced good recoveries within 70 % to 120 % for the target compounds except for AD (some recoveries <70 %) and MFST (some recoveries >120 %) (Fig. 3). Among the five solvents, MeOH generated the best results with ASE. In consideration of the polarity of solvents, MeOH has a strong ability to extract various classes of chemicals in solid samples, but it can also extract other polar impurities such as pigments and humic substances. Those impurities may lead to the difficulty in cleanup and cause matrix interferences during LC-MS/MS analysis of the target compounds, thereby reducing the precision of instrumental analysis. Considering the recoveries of the target compounds, toxicity of solvents, and easiness of extraction processes, USE was chosen as the extraction method for solid samples with EtOAc/MeOH (8/2, *v/v*) as the extraction solvent.

For solid samples, it is often essential to include purification steps before instrumental analysis by LC-MS/MS in order to reduce interferences of impurities. Normal phase and reverse-phase cartridges (e.g., silica or silica gel cartridges, and C_{18} or HLB cartridges) are often applied in solid samples purification as demonstrated in the literature [10, 11, 28]. In this study, self-made silica gel cartridges and Oasis HLB cartridges were tested for the purification of the extracts of solid samples. These two types of cartridges showed good performances, with the recoveries of the target compounds

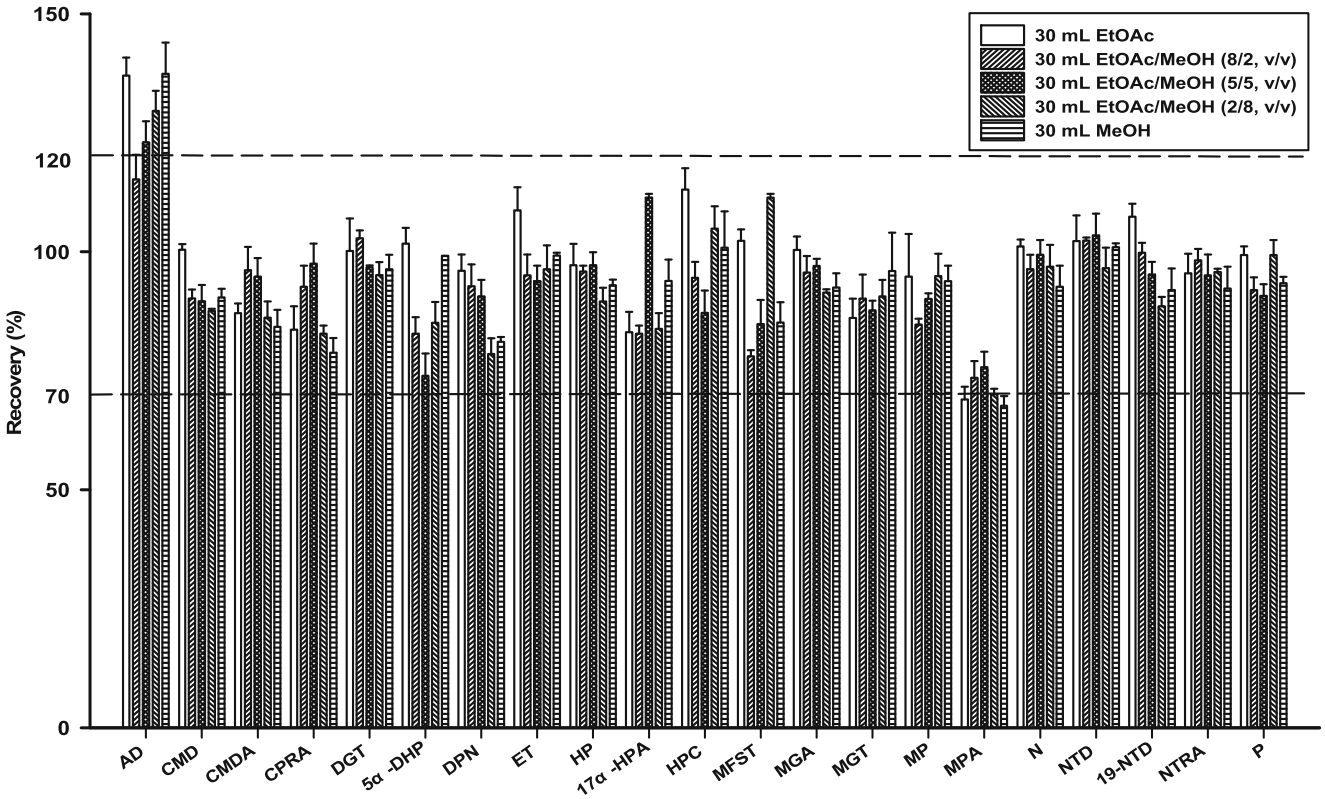

Fig. 2 Recoveries of target compounds from sludge samples by ultrasonic solvent extraction with different elution solvents (*AD*, anordrin; *CMD*, chlormadinone; *CMDA*, chlormadinone acetate; *CPRA*, cyproterone acetate; *DGT*, dydrogesterone; 5α-DHP, 5α-dihydroprogesterone; *DPN*, drospirenone; *ET*, ethynyl testosterone; *HP*, hydroxy progesterone; 17α-HPA, 17α-hydroxyprogesterone acetate; *HPC*, hydroxyprogesterone caproate; *MFST*, mifepristone; *MGA*, melengestrol acetate; *MGT*, megestrol; *MP*, medroxyprogesterone; *MPA*, medroxyprogesterone acetate; *N*, norgestrel; *NTD*, norethynodrel; 19-NTD, 19-norethindrone; *NTRA*, norethisterone acetate; *P*, progesterone; mean±SD; n=3)

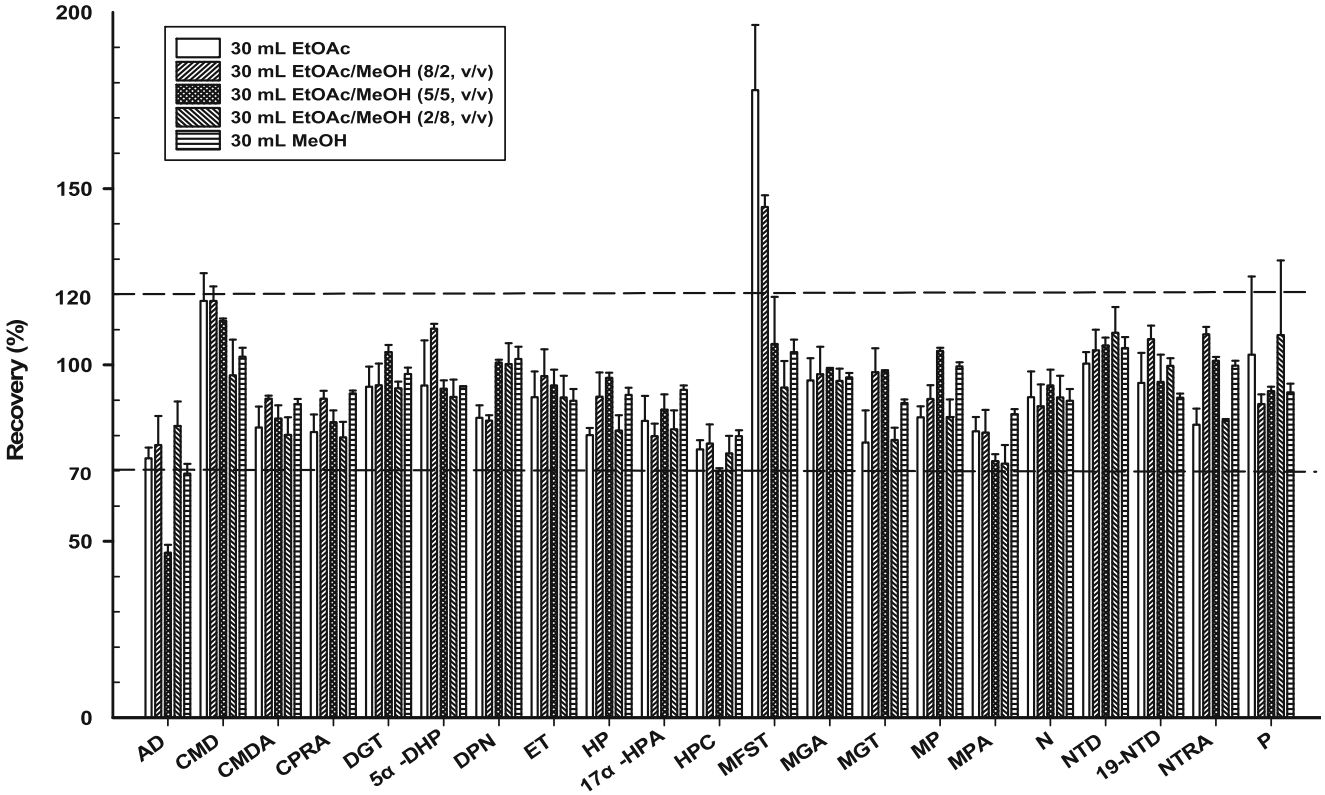

Fig. 3 Recoveries of target compounds from sludge samples by accelerated solvent extraction with different elution solvents (*AD*, anordrin; *CMD*, chlormadinone; *CMDA*, chlormadinone acetate; *CPRA*, cyproterone acetate; *DGT*, dydrogesterone; 5α-DHP, 5α-dihydroprogesterone; *DPN*, drospirenone; *ET*, ethynyl testosterone; *HP*, hydroxy progesterone; 17α-HPA, 17α-hydroxyprogesterone acetate; *HPC*, hydroxyprogesterone caproate; *MFST*, mifepristone; *MGA*, melengestrol acetate; *MGT*, megestrol; *MP*, medroxyprogesterone; *MPA*, medroxyprogesterone acetate; *N*, norgestrel; *NTD*, norethynodrel; 19-NTD, 19-norethindrone; *NTRA*, norethisterone acetate; *P*, progesterone; mean±SD; n=3)

within 70–120 %. Since self-packed cartridges or columns are more economical, self-made silica gel cartridges were selected in this study.

Elution solvent is critical with self-made silica gel cartridges for extract purification. The selected solvent should be able to elute the target compounds effectively, and at the same time, it should also able to minimize or reduce other interfering compounds and matrix substances. Eight different elution solvents (Hex, DCM, EtOAc, and EtOAc/MeOH (9/1, 8/2, 7/3, 6/4 and 5/5, *v/v*)) were tested by spiking with 100 ng each of the analytes to the silica gel cartridges. The results showed that the solvents Hex and DCM gave relatively poor recoveries for all analytes, while the rest six solvents produced relatively satisfactory recoveries. Among the six elution solvents, it was found that EtOAc/MeOH (9/1, *v/v*) gave the best recoveries (81 % to 119 %) for all target compounds. Therefore, EtOAc/MeOH (9/1, *v/v*) was selected as the solvent for purification with silica gel cartridges.

Optimization of instrumental conditions

Agilent Optimizer software was used to optimize LC-MS/MS operating conditions. To obtain a method with both high sensitivity and separation efficiency, different ionization sources, ionization source modes, mobile phases, and flow rates were tested.

The target compounds progestagens have medium polarity, thus both ESI and APCI were tested under both positive and negative ionization modes. In fact, liquid chromatography coupled to electrospray/atmospheric pressure chemical ionization (ESI/APCI) mass spectrometry has also been used to quantitatively determine progestagens in some previous studies [9, 11, 12, 29–31].The standard solutions (5 μL of 1 mg/L for each compound) were analyzed through flow-injection; the results showed contrast effects on the sensitivity with different ionization sources and modes. Better responses of the target compounds were found for ESI as the ionization source in the present study. Under positive ionization mode, the base peak selected for quantification of the selected target compounds was the protonated molecule $[M+H]^+$ while, under negative mode, the corresponding peak was the deprotonated molecule $[M-H]^-$ for each target compound [11]. However, only limited number of the 21 target compounds showed responses under negative ionization mode. Undoubtedly, ESI with positive mode was selected in the present study.

It is commonly accepted that mobile phase composition can influence on the resulting peaks in LC-MS/MS [11, 29]. Different mobile phase A (Milli-Q water, acetic acid, formic acid, and ammonium acetate with different combinations) and mobile phase B (MeOH and acetonitrile) and different flow rates were tested for optimization of the LC mobile phase. For mobile phase A, ammonium acetate at 5 mM together with formic acid at 0.05 % offered the best peak responses and reproducible retention times. Ammonium acetate in mobile phase A can stabilize certain compounds and adjust pH value of the mobile phase; meanwhile, acidification of mobile phase A can provide protons to the target compounds for the generation of protonated molecules, thus improving sensitivity of MS detection. For mobile phase B, MeOH was chosen because it produced higher signal responses and better separation for the target compounds with similar structures, despite that acetonitrile gave better peak shapes than MeOH. Since the flow rate of LC mobile phase can affect peak shape, matrix interference, column pressure, and analysis time, different flow rates (0.30, 0.35, and 0.40 mL/min) were evaluated. The optimized flow rate was found to be 0.35 mL/min, while 0.30 mL/min gave longer run time and 0.40 mL/min led to higher signal suppression. Therefore, the mobile phase used in the present study was Milli-Q water containing 5 mM ammonium acetate and 0.05 % formic acid (*v*/*v*) (A) and MeOH (B) with a flow rate of 0.35 mL/min.

Matrix effect

Matrix effect is a common problem for LC-MS/MS with ESI mode [9, 11, 30, 31], which can be assessed by comparing the matrix extracts spiked with the standard solution to the corresponding standard solution in the mobile phase solvent. The response ratios of lower or higher than 100 % indicate signal suppression or enhancement, respectively. The results shown in Tables S1–S4 (see ESM) indicate that the target compounds in surface water, tap water, influent, effluent, flush water (except 5α-DHP with 65 %, HPC with 54 %), sediment (except 5α-DHP with 57 %), sludge (except AD, 5α-DHP, and HPC with 34 %, 43 %, and 52 %, respectively), and feces (except 5α-DHP, HPC, and MP with 52 %, 61 %, and 130 %, respectively) matrices had not been significantly affected from matrix interferences (matrix effect within 70–120 %) with the optimized methods.

Method validation

Calibration curves were achieved for all target compound standards at eight different concentration levels from 1 to 1,000 μg/L (1, 5, 10, 50, 100, 200, 500, and 1,000 μg/L). A calibration curve was performed in each set of batch, and excellent linearity was achieved with the correlation coefficients higher than 0.98 for all validation batches.

With the optimized methods, good recoveries were obtained for all target compounds in matrix spiked samples of surface water, tap water, wastewater, flush water, sludge, and feces samples (see ESM Tables S1–S4). The method LOD and LOQ were calculated for each target compound based on the S/N near the target peak. The LOQs of the target compounds based on the lowest spiking levels in the surface water, tap water, influent, effluent, flush water, sediment, dewatered sludge, and feces samples were 0.03–0.39, 0.02–0.32, 0.08–2.30, 0.06–0.65, 0.04–1.10 ng/L, and 0.01–0.65, 0.06–2.59, and 0.17–1.62 ng/g (except for AD at 1.75, 0.87, 5.80, 2.96, 3.31 ng/L, 3.55, 12.30, and 6.71 ng/g), respectively. In consideration of complex matrix interferences in the dewatered sludge samples, higher LOQs would be expected due to the increased background noise [11]. It is well-known that some limited methods for determination of the concentration of some progestagens in different environmental matrices were reported; a comparison with the previous results is given in Table 2. Satisfactory results were obtained from the present study. Moreover, the present study presented more systematic progestagens compounds and more diverse environmental matrices, which is essential for environmental risk assessment of various progestagens.

Both intra-day and inter-day precisions were calculated for the UHPLC-MS/MS method. For the intra-day precision, a standard solution (10 μg/L of each compound) was injected successively seven times within a day. The repeatability expressed as the relative standard deviation (RSD) of the seven measured concentrations for each compound was found in the range between 0.6 and 4.0 % for all compounds. For the inter-day, it was obtained from five injections each day of the standard solutions at a concentration of 10 μg/L of each compound, which were carried out on five different days over 1-month interval, and the RSD was less than 5.2 % for all target compounds.

Application to environmental samples

The optimized method for the progestagens was applied to simultaneously analyze 21 target compounds in different environmental matrices. The mean concentrations of the detected compounds are given in Table 3. The results showed that 3, 3, 0, 7, 3, 3, 3, 4, 13, and 6 compounds were detected in Liuxi Reservoir water and sediment, tap water, influent, effluent, and dewatered sludge from Xintang WWTP, upstream and downstream river water near the WWTP, flush water, and feces from swine farm, respectively. Among all the sample matrices, flush water contained the most progestagens with the highest concentrations (total concentrations more than 10,000 ng/L), followed by feces (total concentrations nearly 6,000 ng/g). Previous studies reported that progesterone was detected in piled manure in USA at the concentration of 196 ng/g [20] and progesterone and norgestrel in flush

Table 2 Comparison of the different methods of progestagens in various environmental matrices

Compound	LOD (ng/L or ng/g)	LOQ (ng/L or ng/g)	Recovery (%)	Matrix	Instrument	Reference
AD	0.26–3.68	0.87–12.30	35–141	Eight matrices[a]	LC-MS-MS	This study
CMD	0.03–0.19	0.10–0.63	90–132	Eight matrices	LC-MS-MS	This study
CMDA	0.01–0.13	0.05–0.43	70–100	Eight matrices	LC-MS-MS	This study
CPRA	0.02–0.21	0.06–0.71	77–114	Eight matrices	LC-MS-MS	This study
DGT	0.04–0.69	0.14–2.30	79–108	Eight matrices	LC-MS-MS	This study
5α-DHP	0.03–0.78	0.10–2.59	46–119	Eight matrices	LC-MS-MS	This study
DPN	0.01–0.14	0.02–0.47	76–129	Eight matrices	LC-MS-MS	This study
ET	0.003–0.13, 0.04–0.54	0.01–0.43, 0.13–1.81	63–110, 90–146	Eight matrices, four matrices[b]	LC-MS-MS, LC-MS-MS	This study, [11]
HP	0.01–0.34, –, –	0.03–0.13, 0.10–0.30, 0.10–0.30	86–120, –, 81–84	Eight matrices, influent, effluent, influent, effluent	LC-MS-MS, LC-MS-MS, LC-MS-MS	This study, [9], [33]
17α-HPA	0.01–0.12	0.02–0.43	74–108	Eight matrices	LC-MS-MS	This study
HPC	0.02–0.33	0.06–1.12	38–104	Eight matrices	LC-MS-MS	This study
MFST	0.01–0.62	0.02–2.05	62–120	Eight matrices	LC-MS-MS	This study
MGA	0.01–0.05	0.02–0.17	79–111	Eight matrices	LC-MS-MS	This study
MGT	0.04–0.48, –	0.14–1.61, 0.70	85–128, 80	Eight matrices, drinking water	LC-MS-MS, LC-MS-MS	This study, [34]
MP	0.01–0.12, 0.04–0.38, –, –	0.04–0.41, 0.13–1.28, 8–95, 1.60	90–129, 100–140, 21–59, 106	Eight matrices, four matrices, dairy wastewater, drinking water	LC-MS-MS, LC-MS-MS, GC-MS, LC-MS-MS	This study, [11], [20], [34]
MPA	0.01–0.06, –, –	0.03–0.21, 0.02–0.10, 0.01–0.16	70–113, –, 82–86	Eight matrices, influent, effluent, influent, effluent	LC-MS-MS, LC-MS-MS, LC-MS-MS	This study, [9], [33]
N	0.02–0.53, –, 0.03–0.90, –	0.06–1.77, 0.08–0.30, 0.10–2.99, 0.24–0.90	63–109, –, 88–142, 78–83	Eight matrices, influent, effluent, four matrices, influent, effluent	LC-MS-MS, LC-MS-MS, LC-MS-MS, LC-MS-MS	This study, [9], [11], [33]
NTD	0.01–0.19	0.04–0.65	75–121	Eight matrices	LC-MS-MS	This study
19-NTD	0.02–0.24, 0.02–1.92, –	0.07–0.79, 0.08–6.39, –	72–107, 54–118, 95	Eight matrices, four matrices, effluent	LC-MS-MS, LC-MS-MS, GC-MS	This study, [11], [35]
NTRA	0.02–0.24	0.08–0.80	87–111	Eight matrices	LC-MS-MS	This study
P	0.01–0.07, –, 0.05–0.42, –, –, –, –, –, –, –, –	0.03–0.24, 0.13–0.50, 0.17–1.39, 0.39, 17–210, 0.13, –, –, 0.30, 0.02–0.50, 0.02	79–110, –, 92–119, 108, 92–130, 67, 66, 100, 91, 83–100, 81	Eight matrices, influent, effluent, four matrices, river water, dairy wastewater, soil, sediment, effluent, effluent, influent, effluent, drinking water	LC-MS-MS, LC-MS-MS, LC-MS-MS, LC-MS-MS, GC-MS, LC-MS-MS, LC-MS, GC-MS, LC-MS, LC-MS-MS, LC-MS-MS	This study, [9], [11], [12], [20], [30], [31], [33], [34], [35], [36]

AD anordrin, *CMD* chlormadinone, *CMDA* chlormadinone acetate, *CPRA* cyproterone acetate, *DGT* dydrogesterone, *5α-DHP* 5α-dihydroprogesterone, *DPN* drospirenone, *ET* ethynyl testosterone, *HP* hydroxy progesterone, *17α-HPA* 17α-hydroxyprogesterone acetate, *HPC* hydroxyprogesterone caproate, *MFST* mifepristone, *MGA* melengestrol acetate, *MGT* megestrol, *MP* medroxyprogesterone, *MPA* medroxyprogesterone acetate, *N* norgestrel, *NTD* norethynodrel, *19-NTD* 19-norethindrone, *NTRA* norethisterone acetate, *P* progesterone, *LOD* method limit of detection, *LOQ* method limit of quantitation

[a] Eight matrices: tap water, surface water, WWTP influent, WWTP effluent, flush water, sediments, sludge, feces

[b] Four matrices: surface water, WWTP influent, WWTP effluent, sludge

Table 3 Concentrations of target compounds in liquid and solid matrix samples from different sources

Compound	Liuxi River reservoir (n=3)[a]		Tap water (n=3)[a]	Zengcheng WWTP (n=3)[a]					Shunfeng swine farm (n=3)[a]	
	Water (ng/L)	Sediment (ng/g)	(ng/L)	Influent (ng/L)	Effluent (ng/L)	Dewatered sludge (ng/g)	Upstream river water (ng/L)	Downstream river water (ng/L)	Flush water (ng/L)	Feces (ng/g)
AD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CMD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CMDA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CPRA	ND	ND	ND	ND	ND	ND	ND	ND	127±4.8	ND
DGT	ND	ND	ND	35.1±3	ND	5.6±0.3	ND	9.6±0.4	2,188±9.5	1,704±7.8
5α-DHP	ND	5.9±0.6	ND	24.3±2	ND	11.8±1.6	3.9±0.3	ND	871±6.4	1,069±8.9
DPN	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ET	ND	ND	ND	ND	ND	ND	ND	ND	23.8±4.4	ND
HP	ND	ND	ND	ND	ND	ND	ND	1.6±0.2	217±7.4	27.3±5.2
17α-HPA	ND	ND	ND	0.7±0.1	ND	ND	ND	ND	137±0.43.8	ND
HPC	ND	1.7±0.1	ND	ND	ND	ND	ND	ND	51.1±5.0	ND
MFST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MGA	0.6±0.1	ND	ND	3.0±0.1	1.2±0.1	ND	ND	ND	ND	ND
MGT	ND	ND	ND	ND	ND	ND	ND	ND	397±3.2	ND
MP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MPA	ND	ND	ND	2.4±0.1	0.9±0.1	ND	ND	ND	330±7.7	21.0±4.3
N	1.1±0.1	ND	ND	5.5±0.3	ND	ND	ND	ND	642±8.1	1,315±6.4
NTD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
19-NTD	ND	ND	ND	ND	ND	ND	3.6±0.2	3.7±0.2	143±3.3	ND
NTRA	ND	ND	ND	ND	ND	ND	ND	ND	2,134±8.5	ND
P	1.7±0.2	3.4±0.1	ND	10.1±0.3	2.9±0.1	6.0±0.3	1.2±0.1	2.5±0.1	5,024±6.2	1,952±10.7

AD anordrin, *CMD* chlormadinone, *CMDA* chlormadinone acetate, *CPRA* cyproterone acetate, *DGT* dydrogesterone, *5α-DHP* 5α-dihydroprogesterone, *DPN* drospirenone, *ET* ethynyl testosterone, *HP* hydroxy progesterone, *17α-HPA* 17α-hydroxyprogesterone acetate, *HPC* hydroxyprogesterone caproate, *MFST* mifepristone, *MGA* melengestrol acetate, *MGT* megestrol, *MP* medroxyprogesterone, *MPA* medroxyprogesterone acetate, *N* norgestrel, *NTD* norethynodrel, *19-NTD* 19-norethindrone, *NTRA* norethisterone acetate, *P* progesterone, *ND* no detection

[a] Mean (%)±standard deviation (%) (n=3)

samples from a swine farm in Guangxi (China) with their concentrations above 10,000 ng/L [32]. In the WWTP, influent contained the most progestagens (total concentrations up to 81 ng/L), followed by dewatered sludge (total concentrations up to 23 ng/g). A previous study reported nine compounds of progestagens (total concentrations up to 57 ng/L in influent, 8 ng/L in effluent, and 13 ng/g in dehydrated sludge samples) in a municipal WWTP in Beijing, China [10]. Moreover, the results from the present study confirmed that swine farms and WWTPs are the main sources of progestagens in the environment [12, 32]. Comparing with influent, effluent contained less and lower target compounds (total concentrations 5 ng/L), indicating that the treatment processes of the WWTP could effectively remove or transform progestagens. The concentrations of the detected compounds in the river downstream (total concentrations up to 17 ng/L) were higher than those in the river upstream (total concentrations up to 9 ng/L) and even the effluent from WWTP. It is mainly due to discharge of some other pollution sources, such as untreated sewage near the river [11]. The concentrations of the detected compounds in the surface water from Liuxi Reservoir were found to be at trace levels around 1 ng/L. Fortunately, there was no target compound detected in the tap water

Conclusion

A sensitive and reliable analytical method was developed for simultaneous determination of 21 progestagens in environmental samples by UHPLC-MS/MS with ESI under positive ionization mode. The method involved solid-phase extraction for liquid samples and ultrasonic extraction for solid samples followed by purification with self-made silica gel cartridges. The developed method also had some advantages in terms of simplicity and cost, since it can perform the extraction and purification for large volume liquid samples and solid samples in a relatively short time. It has been successfully applied in

the analysis of progestagens in various environmental samples. The most frequently detected compound was progesterone with its highest levels being found in flush water and feces of the swine farm, followed by synthetic compounds DGT, 5α-DHP, N, and NTRA at remarkable levels. This developed analytical method provided a robust tool for the simultaneous screening and determination of progestagens in various environment matrices.

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