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Trace analysis of androgens and progestogens in environmental waters by ultra-performance liquid chromatography–electrospray tandem mass spectrometry

Hong Chang[a], Shimin Wu[a], Jianying Hu[a,*], Mari Asami[b], Shoichi Kunikane[b]

[a] College of Urban and Environmental Sciences, Peking University, Beijing 100871, China
[b] Department of Water Supply Engineering, National Institute of Public Health, Saitama 351-0197, Japan

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ABSTRACT

A sensitive ultra-performance liquid chromatography–electrospray tandem mass spectrometry method, combined with solid-phase extraction and silica cartridge cleanup, was established for nine androgens (androstenedione, 19-nor-4-androstene-3,17-diol, androsterone, epiandrosterone, testosterone, methyltestosterone, trenbolone, nandrolone, stanozolol) and nine progestogens (progesterone, 17α-hydroxyprogesterone, 21α-hydroxyprogesterone, 6α-methyl-hydroxyprogesterone, 17α,20β-dihydroxy-4-pregnene-3-one, megestrol acetate, norethindrone, norgestrel, medroxyprogesterone acetate) in environmental waters. For the various water matrices considered, the overall method recoveries were from 78 to 100%, and no apparent signal suppression was found. The method detection limits for the eighteen analytes in the influent, effluent and surface water samples were 0.20–50, 0.04–20 and 0.01–12 ng/L, respectively. This method was used to analyze the residual androgens and progestogens in the wastewater and surface water samples from Japan, and ten analytes (0.03 (medroxyprogesterone acetate)–1441 ng/L (androsterone)) were detected in the wastewater samples, and four analytes (0.06 (progesterone)–0.46 ng/L (androstenedione)) were detected in the surface water samples.

1. Introduction

Considerable attention had been focused on the occurrence of estrogenic steroid hormones in the environment since an initial report showed that the exposure of fish to municipal wastewater effluents resulted in the feminization of fish at concentrations as low as 1 ng/L [1]. Recent studies have documented the masculinization of fish after their exposure to androgens at similarly low concentrations [2,3], and numerous progestogens, along with certain androgens as hormonal odorants and reproductive pheromones have also been shown to affect the reproductive physiology and behavior in many fish species at ng/L or even pg/L levels [4–6]. Therefore, the presence of androgens and progestogens in the environment should deserve greater attention.

A broad number of natural and synthetic androgens and progestogens have been used in human and veterinary therapy, or as growth promoters, and they can be discharged into the aqueous environment via sewage treatment plants (STPs). Therefore, there has been a need for developing a sensitive and reliable method to analyze the broad number of these compounds in various water matrices in order to assess their environmental risk. Gas chromatography–mass or tandem mass spectrometry [GC–MS(/MS)] has been used to analyze two androgens and one or two progestogens in the wastewater effluents [7] or surface water samples [8] after their derivatization. However, the sample derivatization for the wide range of androgens and progestogens proved complicated. Not all androgens and progestogens, such as stanozolol (an androgen), were able to be derivatized [9]. LC–MS(/MS) is an alternative method due to its sensitivity and specificity, without any need for derivatization, and it has been used to analyze androgens [10] or progestogens [11,12] in wastewater and surface water samples. However, both GC–MS(/MS) and LC–MS(/MS) methods were all targeted for less than four progestogens or five androgens. In addition, although LC–MS(/MS) has been viewed as a potential method for analyzing a broad range of these compounds, the matrix interference has proven to be a general problem even for the LC–MS/MS system, as exemplified by the signal suppression of progesterone (up to 38%) in the surface water samples [13].

In this study, we developed a sensitive and specific method for simultaneously analyzing nine androgens and nine progestogens in wastewater using solid-phase extraction (SPE) and ultra-

* Corresponding author. Tel.: +86 10 62765520; fax: +86 10 62765520.
E-mail address: hujy@urban.pku.edu.cn (J. Hu).

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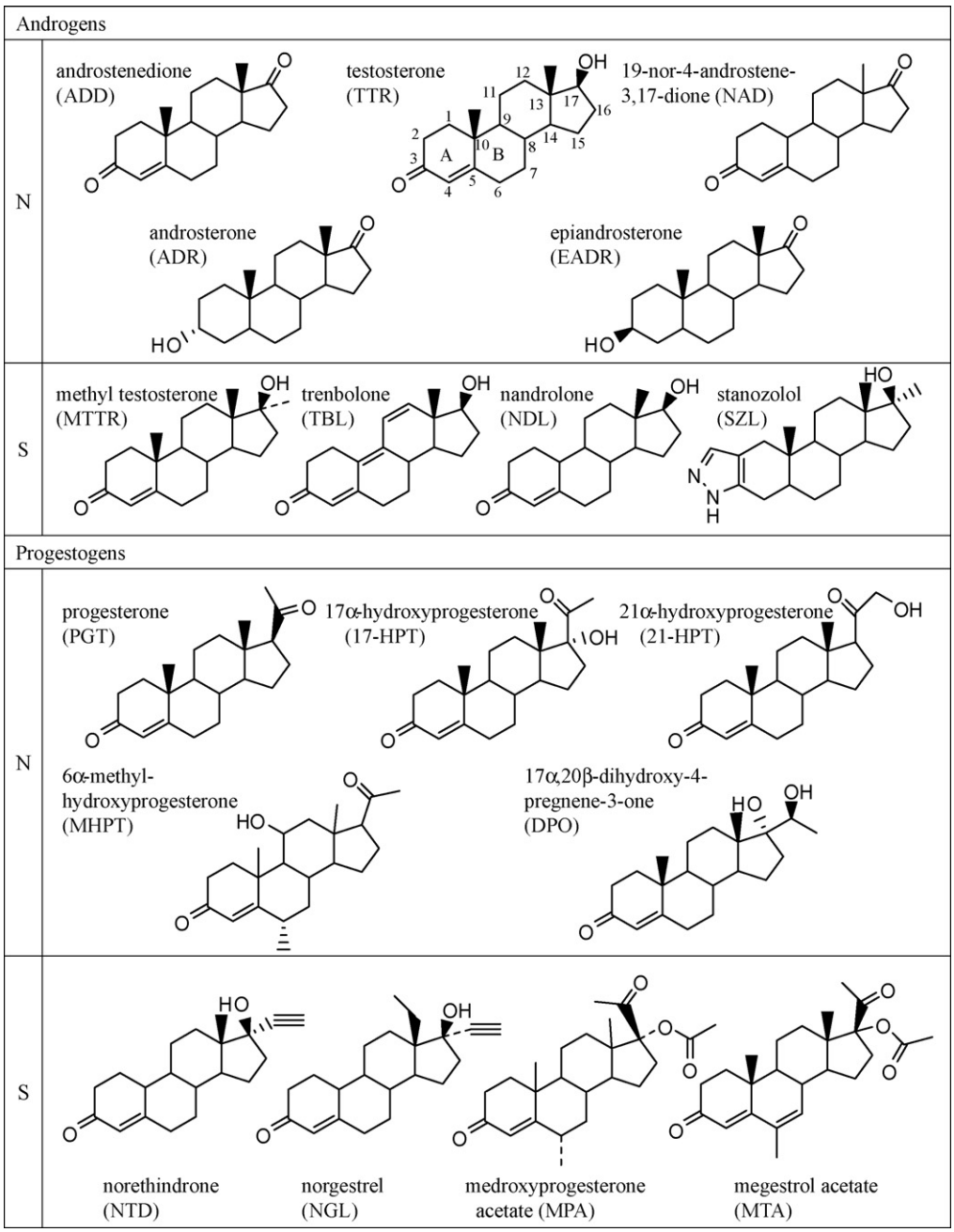

Fig. 1. Structure of target androgens and progestogens. N: natural steroid; S: synthetic steroid.

performance (UP)-LC–MS/MS analysis, where a silica cartridge was used in the sample cleanup. The target sex hormones (Fig. 1) were chosen from natural and synthetic androgens and progestogens which have been detected [7–13] or could be potentially present in the environment. Finally, this developed method was applied to the analysis of these compounds in wastewater and surface water samples.

2. Experimental

2.1. Materials

19-Nor-4-androstene-3,17-diol (NAD), trenbolone (TBL), nandrolone (NDL), androstenedione (ADD), norethindrone (NTD), 17α-hydroxyprogesterone (17-HPT), testosterone (TTR), 21α-hydroxyprogesterone (21-HPT), norgestrel (NGT), 17α,20β-dihydroxy-4-progegnen-3-one (DPO), methyltestosterone (MTTR), epiandrosterone (EADR), stanozolol (SZL), 6α-methylhydroxyprogesterone (MHPT), megestrol acetate (MTA), medroxyprogesterone acetate (MPA), progesterone (PGT), androsterone (ADR), [$^{13}C_2$]ethynyl-NTD, [$^{13}C_2$]TTR, [$^{2}H_6$]NGT (NGT-d_6) and [$^{2}H_9$]PGT (PGT-d_9) were purchased from Sigma (St Louis, MO, USA). Formic and acetic acids were of analytical grade (Wako, Saitama, Japan). Methanol, acetonitrile, ethyl acetate, hexane, and dichloromethane were all of HPLC grade purchased from Fisher Chemical (Japan). HPLC-grade water was prepared using a Milli-Q RC apparatus (Millipore, Bedford, MA, USA).

2.2. Sample collection

Influent and final effluent samples were collected in 5 L amber glass bottles, which were previously washed with methanol and distilled water, from two STPs in Saitama, Japan on 27 July 2007. Both STPs receive mainly domestic wastewater, and are operated with primary, aerobic biological and secondary treatments. Four surface water samples were collected from the Koyama river basin, which is known to be a major farming area in the Saitama prefecture, Japan. All samples were collected as grab samples and were extracted on the same day after being filtered by a glass microfiber filter GF/F 0.7 μm (Whatman, Maidstone, UK).

2.3. Sample extraction and cleanup

All target androgens and progestogens were extracted simultaneously using one Oasis HLB cartridge (6 mL, 500 mg, Waters, Milford, MA, USA). The cartridge was preconditioned with 6 mL of ethyl acetate, 6 mL of acetonitrile and 12 mL of distilled water. The influent samples (0.5 L), the effluent samples (1 L) and the river water samples (2 L), spiked with 2.5 ng of four surrogate standards, were extracted through the HLB cartridges at a flow rate of 5–10 mL/min. The cartridges were rinsed with 10 mL of distilled water, and then were dried under a flow of nitrogen. The target androgens and progestogens were then eluted with 15 mL of ethyl acetate. The extracts were dried and re-dissolved in 0.2 mL of ethyl acetate and 1.8 mL of hexane. The mixed solutions were applied to the silica cartridges (3 mL, 500 mg, Waters), which had been preconditioned with 4 mL of water-saturated ethyl acetate and 4 mL of hexane/ethyl acetate (90:10, v/v). After the cartridges were rinsed with 3 mL of hexane/ethyl acetate (90:10, v/v), the target hormones were eluted with 3 mL of hexane/ethyl acetate (38:62, v/v). The eluate was then dried and reconstituted, respectively, with 0.5 mL of methanol for the UPLC–electrospray ionization (ESI) MS/MS analysis.

2.4. LC–MS/MS

The LC apparatus was an Acquity Ultra Performance LC (Waters). All androgens and progestogens were separated using a Waters Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm particle size). To compare with the conventional HPLC separation, a Waters Symmetry C18 column (150 × 2.1 mm, 5 μm particle size) was also used in this study. The UPLC column was maintained at 40 °C at a flow rate of 0.3 mL/min, and the injection volume was 5 μL. Methanol (A) and water, containing 0.1% formic acid, were used as the mobile phases. The gradient conditions for UPLC column were initiated with 60% A, followed by a linear increase to 65% A in 2.5 min. After it was increased to 70% in 3.5 min, the mobile phase A was increased sharply to 100% in 0.1 min, and then was held for 1 min. The gradient conditions for HPLC column were linearly increased from 60 to

Table 1
Multi-selected reaction monitoring (MRM) conditions of the target androgens and progestogens

Compound	MRM transition	Cone voltage (V)	Collision energy (eV)	Dwell time (s)	Segment period (min)
NAD	273 > 109	33	24	0.1	2.0–2.6
	273 > 197		18		
TBL	271 > 199	37	21		
	271 > 253		19		
NDL	275 > 109	35	21		
	275 > 257		15		
ADD	287 > 97	33	22		
	287 > 109		24		
NTD	299 > 231	31	20		
	299 > 109		26		
[$^{13}C_2$]Ethynyl-NTD	301 > 109	31	26	0.05	2.6–3.8
17-HPT	331 > 97	33	26		
	331 > 109		24		
TTR	289 > 97	33	22		
	289 > 109		22		
[$^{13}C_2$]TTR	291 > 99	33	20		
21-HPT	331 > 97	33	26		
	331 > 109		24		
NGT	313 > 245	31	16		
	313 > 109		26		
NGT-d_6	319 > 114	33	24		
DPO	333 > 97	33	24		
	333 > 109		30	0.05	3.6–4.6
MTTR	303 > 97	33	23		
	303 > 109		23		
EADR	291 > 255	25	12		
	291 > 273		10		
SZL	329 > 81	47	40		
	329 > 95		40		
MHPT	345 > 123	39	24	0.1	4.6–5.1
	345 > 97		24		
MTA	385 > 267	25	20		
	385 > 325		14		
MPA	387 > 327	29	14		
	387 > 285		18		
PGT	315 > 97	32	24	0.1	5.1–5.8
	315 > 109		24		
PGT-d_9	324 > 100	33	22		
ADR	291 > 255	20	12	0.25	5.9–6.5
	291 > 273		10		

80% A in 10 min, then increased to 100% A in 1.5 min and kept for 5 min.

Mass spectrometry was performed using a Waters TQ detector which was operated with ESI in the positive ion (PI) mode. The detection conditions of the mass spectrometer were as follows: capillary voltage, 2.5 kV; source temperature, 120 °C; desolvation temperature, 450 °C; source gas flow, 50 L/h; and desolvation gas flow, 900 L/h. Finally, the data acquisition was performed under time-segmented conditions based on the chromatographic separation of the target compounds to maximize sensitivity of detection (Table 1).

2.5. Method validation

Identification of the target androgens and progestogens was accomplished by comparing the retention time (within 2%) and the ratio (within 20%) of the two selected MRM ion transition with those of standards. To automatically correct the loss of analytes during the sample preparation and the matrix-induced change in ionization, and to compensate for variations in the instrument response from injection to injection, [$^{13}C_2$]TTR, [$^{13}C_2$]ethynyl-NTD, NGT-d_6 and PGT-d_9 were used as surrogate standards in this study. Carbon-13 is a naturally occurring isotope of carbon, and the signal of surrogate [$^{13}C_2$]TTR may be affected by the naturally occurring carbon-13 of TTR at high levels. In this study, we found that the abundance of the naturally occurring [$^{13}C_2$]TTR in TTR standard resolution even at 100 μg/L level, largely higher than the concentration in real sample extract (<20 μg/L in this study), was only 6.1% of the signal intensity of sample extract spiked by surrogate [$^{13}C_2$]TTR at 5 μg/L level. This result indicates that [$^{13}C_2$]TTR is an effective surrogate standard.

All equipment rinses were done with methanol to avoid sample contamination, and laboratory blanks were analyzed to assess potential sample contamination. Recovery experiments were done by spiking the standard solutions to an influent, an effluent and a surface water sample. Analyte addition was made with the criterion of at least three times the original concentration that was determined prior to the fortification experiment.

3. Results and discussion

3.1. LC–MS/MS analysis

The UPLC system using 1.7 μm particle size columns can be expected to give a high sample throughput. In this study, although a very slow gradient method was applied, the very sharp peaks were obtained with peak width of 10–15 s at base giving a peak capacity for the 6-min separation of approximately 15–40. In comparison, using a 5 μm particle size C18 column, the peak width increased to 50–60 s at base giving a total peak capacity of approximately 8–12 for the 10-min separation (for experimental details on this 5 μm column see Section 2).

The optimal UPLC–MS/MS conditions are important for the unequivocal identification of androgens and progestogens at very low levels in the environmental samples. Since the ESI is largely dependent on the solvent conditions, the mobile phase composition and the additive were investigated. In this study, a methanol/water mixture containing formic acid was used since this mobile phase composition produced a three- to four-fold increase in the signal intensity, as compared to the acetonitrile/water containing acetic acid for some of the androgens (e.g., ADR and EADR).

The androgens and progestogens were analyzed by MS/MS in the multiple-reaction monitoring (MRM) mode. The two most abundant MRM transitions, cone voltage and collision energies, were optimized for each analyte by infusing the standard solutions into the mass spectrometer (Table 1). All the precursor ions were protonated molecular ions ([M + H]$^+$). All eighteen androgens and progestogens except for ADR, EADR and SZL were 4-ene-3-one-containing steroids, and thus a pattern of common product ions were found in their mass spectra. In the spectra of ADD, TTR, PGT, MTTR, DPO, 17-HPT and 21-HPT, the product ions at m/z 97 and 109 were generated due to the cleavages in the A and B rings. The product ions at m/z 97 and 123 for MHPT with a methyl-substitution at C-6 were also explained by cleavages in the A and B rings. No ion at m/z 97 was obtained for NTD, NGT, NAD and NDL without a methyl-substitution at C-10, indicating that this substitution was important for the cleavage in the A ring. In the case of MPA and MTA, the most abundant product ions were at m/z 327 and 285,

Table 2
Recoveries (%) and method detection limits (MDLs, ng/L) in various water matrices

Compound	Recovery (%) ± RSD (%)			MDL (ng/L)			
	Influent	Effluent	Surface water	Distilled water	Influent	Effluent	Surface water
NAD	82 ± 3.8	87 ± 8.0	88 ± 3.2	0.25	0.80	0.40	0.15
TBL	78 ± 1.7	88 ± 2.4	84 ± 5.4	0.50	0.50	0.30	0.10
NDL	83 ± 2.8	89 ± 3.5	82 ± 4.2	0.17	2.4	1.2	0.10
ADD	80 ± 4.2	91 ± 8.1	85 ± 3.6	0.17	2.5	1.2	0.06
NTD	78 ± 6.3	82 ± 3.6	79 ± 7.6	0.40	1.2	0.60	0.30
[$^{13}C_2$]Ethynyl-NTD	80 ± 5.6	79 ± 7.1	77 ± 4.3	0.40	1.2	0.60	0.30
17-HPT	81 ± 7.1	84 ± 2.6	84 ± 6.4	0.10	0.30	0.20	0.10
TTR	84 ± 2.4	87 ± 5.6	81 ± 4.1	0.1	0.20	0.12	0.06
[$^{13}C_2$]TTR	80 ± 3.6	82 ± 2.9	79 ± 4.8	0.1	0.20	0.12	0.06
21-HPT	85 ± 8.2	92 ± 6.4	86 ± 8.4	0.10	0.30	0.20	0.10
NGT	80 ± 5.3	83 ± 7.8	78 ± 5.4	0.30	0.90	0.48	0.24
NGT-d_6	78 ± 7.2	80 ± 5.5	80 ± 4.7	0.30	0.90	0.48	0.24
DPO	79 ± 3.7	85 ± 4.9	89 ± 3.9	0.13	2.3	1.0	0.50
MTTR	81 ± 9.1	83 ± 8.6	84 ± 8.1	0.15	0.80	0.40	0.20
EADR	82 ± 5.2	85 ± 3.5	82 ± 4.4	2.5	50	20	12
SZL	86 ± 10	93 ± 7.9	86 ± 8.2	0.12	0.24	0.10	0.06
MHPT	83 ± 6.7	86 ± 7.6	84 ± 5.2	0.10	0.20	0.10	0.05
MTA	85 ± 2.8	90 ± 5.8	82 ± 6.3	0.04	0.12	0.06	0.03
MPA	82 ± 2.4	86 ± 5.4	82 ± 7.9	0.16	0.16	0.04	0.01
PGT	86 ± 8.2	100 ± 12	83 ± 10	0.05	0.50	0.26	0.02
PGT-d_9	89 ± 7.0	98 ± 10	85 ± 7.9	0.05	0.50	0.26	0.02
ADR	82 ± 9.8	86 ± 7.2	82 ± 5.8	1.0	20	10	5.0

325 and 267, respectively, due to the subsequent cleavage at C-17. While for one pair of isomers of ADR and EADR with hydroxyl bonds at C-7, the major product ions (m/z 255 and 273) were the result of a subsequent loss of H_2O from the protonated molecule.

3.2. Matrix effects and method performance

In the LC–MS (/MS) analysis, a general and well-known problem is the matrix effects (e.g., signal suppression and isobaric interference) due to co-eluting interferences, which considerably reduces the detection sensitivity and reliability. Vanderford et al. [13] reported a large degree of signal suppression (38%) for PGT in the surface water, as measured by ESI–MS/MS. The most direct means for obtaining a maximum sensitivity and signal reproducibility was through the reduction of the matrix components prior to the instrumental detection, applying an improved sample cleanup. In this study, we simultaneously extracted eighteen androgens and progestogens and four surrogate standards ([$^{13}C_2$]TTR, [$^{13}C_2$]ethynyl-NTD, NGT-d_6 and PGT-d_9) from water samples by a HLB cartridge, and then used a silica cartridge to purify the extract. The results of the spiking experiments at various water matrices were listed in Table 2, and the overall method recoveries for the target androgens, progestogens and surrogate standards were between 78 and 100%, with a RSD less than 12%, and no apparent signal suppression was found as shown in Fig. 2. Fig. 3 shows the cleanup effectiveness using silica cartridge. It was found that the signal/noise (S/N) ratios for the four surrogate standards were largely improved by the cleanup procedure. Especially while distinguishable peaks of [$^{13}C_2$]TTR and [$^{13}C_2$]ethynyl-NTD were found in the cleanup sample (Fig. 3a), no detectable signal was obtained in the chromatogram of the same sample without cleanup (Fig. 3b).

Throughout the whole determination procedure, no contamination of blanks was detected. Calibration curves were constructed for ADR and EADR from 1.0 to 2500 μg/L (standard concentration levels at 1.0, 5.0, 20, 100, 500, 1000, 2500 μg/L) and for other sixteen analytes from 0.1 to 200 μg/L (standard concentration levels at 0.1, 0.5, 2, 10, 25, 100 and 200 μg/L), and the coefficients of determination were typically greater than 0.99. Ten replicate determinations of 1 μg/L of standard aqueous solutions were carried out on the same day under the optimum conditions to determine the run-to-run precision of UPLC–ESI–MS/MS analysis. The RSD was typically less than 9%. During the recovery experiment, one spiked influent sample was analyzed in 15-day period and the typical RSD was lower than 12% by day-by-day replicate determinations. Since many target analytes were expected to occur in three types of water matrices, the estimation of the method detection limits (MDLs) was based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyzing field samples and on a minimal value of

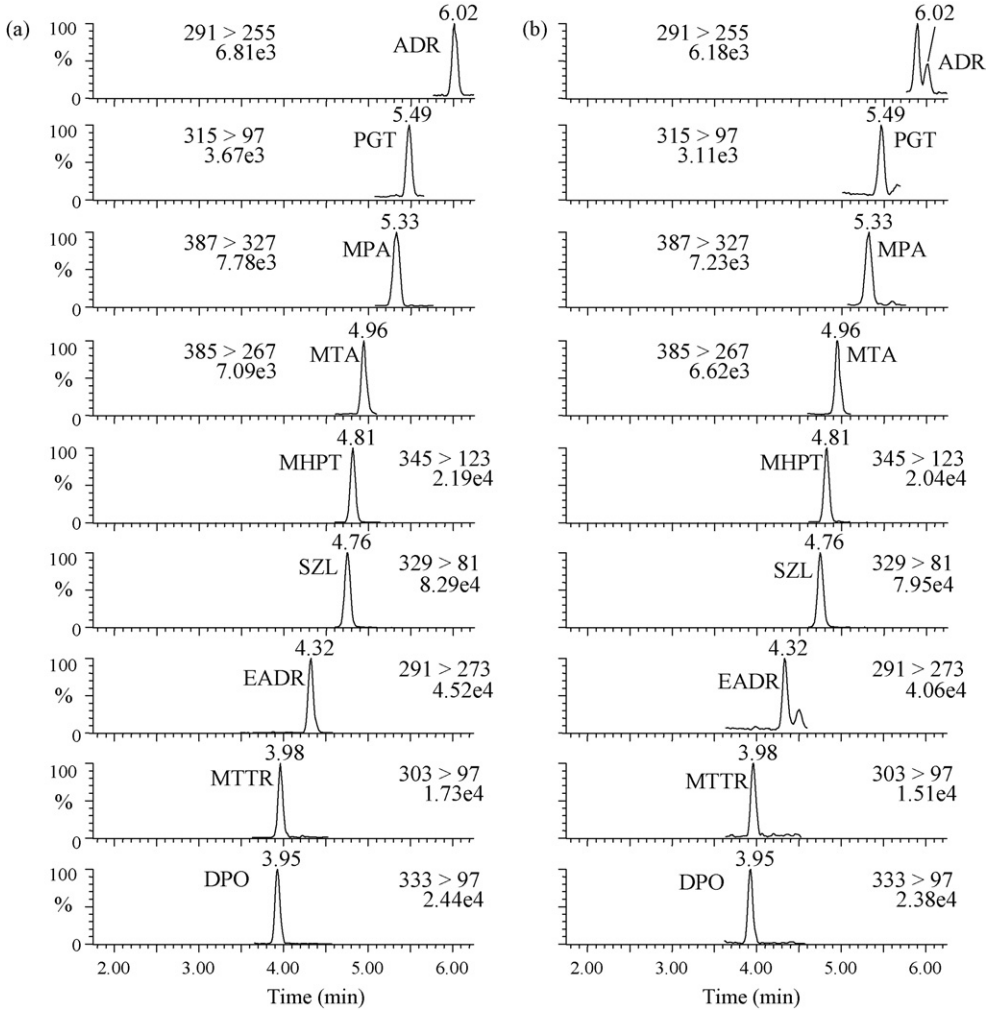

Fig. 2. UPLC–MS/MS MRM chromatograms of eighteen androgens and progestogens (1–100 μg/L) in a standard (a) and a wastewater sample extract (b).

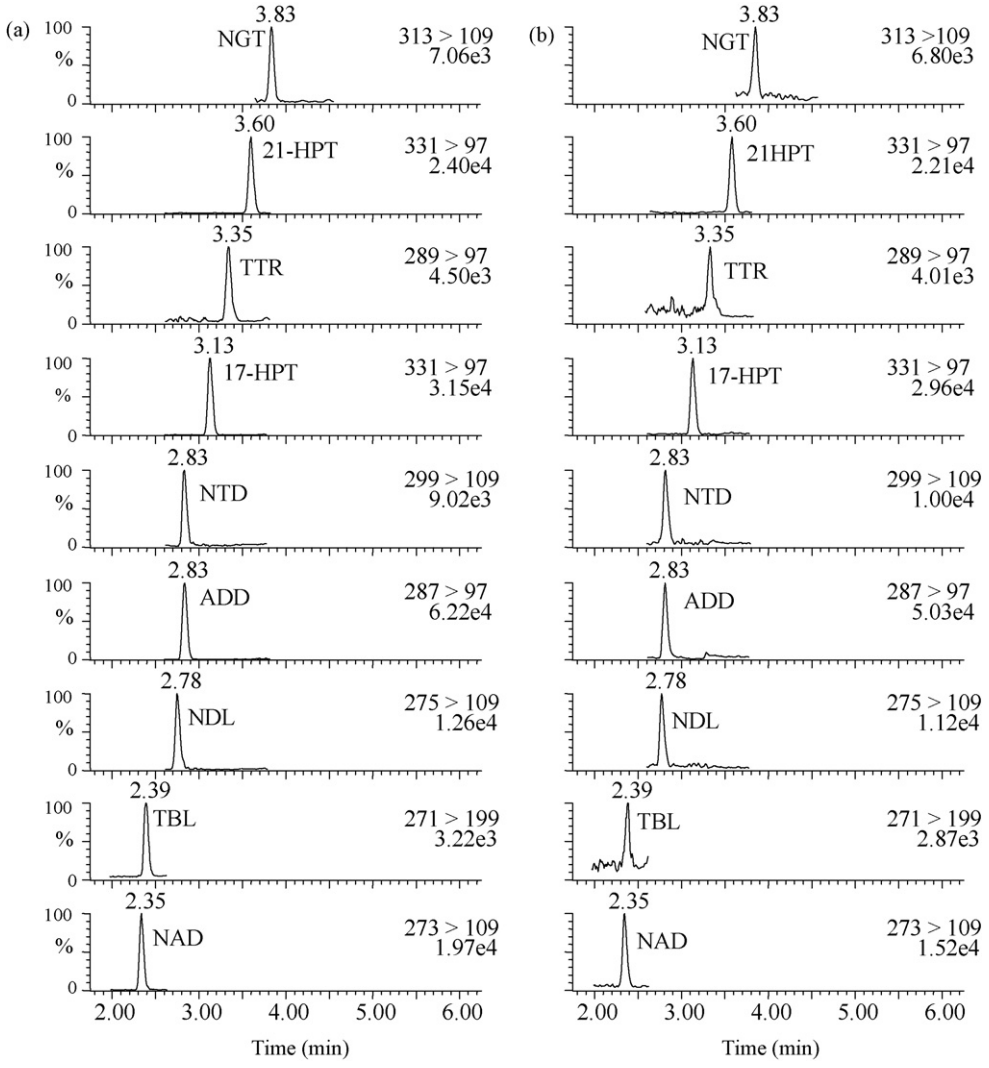

Fig. 2. (*Continued*).

signal-to-noise of 3. For the noncontaminated samples were spiked at concentration ranged from 0.005 to 100 ng/L using a mixture of standard resolution, and the MDLs in distilled water were in the range of 0.05–0.5 ng/L, except for ADR (1 ng/L) and EADR (2.5 ng/L) (Table 2). The MDLs for the target analytes in the influent, effluent and surface water samples were 0.20–50, 0.04–20 and 0.01–12 ng/L, respectively (Table 2). These higher MDLs, when compared with those in the distilled water, were caused by the elevating chromatogram baseline due to some isobaric interferences existing in the extract samples, since the good overall method recoveries in the various water matrices as described above suggested no apparent signal suppression in this study.

3.3. Environmental samples

Two influent, two effluent and four surface water samples were analyzed in duplicate by this method. The mean concentrations of the detected analytes were reported in Table 3. Matrix spikes ($n = 3$) in one influent and one surface water sample were also extracted, and the mean recoveries were also shown in Table 3. Fig. 4 showed the MRM UPLC–MS/MS chromatograms of the extracts from a STP influent. Of the eighteen target androgens and progestogens, eight (ADD, ADR, TTR, EADR, 21-HPT, DPO, MPA and PGT) were detected in the influent samples (1.7 (DPO)-1441 ng/L (ADR)), while seven (ADD, 21-HPT, DPO, MHPT, MTA, MPA and PGT) were detected in the effluent samples (0.03 (MPA)-5.3 ng/L (ADD)). Comparing the concentrations in the influents with that of the effluents, we found that the reduction in the concentrations of ADR, EADR and TTR was almost 100% and of ADD, 21-HPT, MPA and PGT varied from 82 to 95%. It was interesting that the reduction of DPO was limited to 17–54%. Although these removals are estimated by analyzing grab samples, the data of ADD, TTR and PGT agree with the previous study, in which these compounds are found to be removable during biological wastewater treatment [14]. It should be noted that MHPT (0.24 and 1.6 ng/L) and MTA (0.35 ng/L) were detected in the effluents despite the lack of any detection in the influents. This may be due to the fact that some kind of biological conversion occurred in the wastewater treatment process exemplified by the deconjugation of estrogen glucuronidates and sulfates in STP [15], and further investigation is necessary. In the surface water samples, ADD, DPO, PGT and NAD were detected with concentrations of 0.38 ± 0.08, 0.15 ± 0, 0.22 ± 0.04 and 0.07 ± 0.01 ng/L, which exceeded the olfactory detection thresholds for pheromones. These may be high enough to elicit pheromonal responses in certain species of fish when the hormones originate in effluent or even untreated wastewater [4,5,16,17].

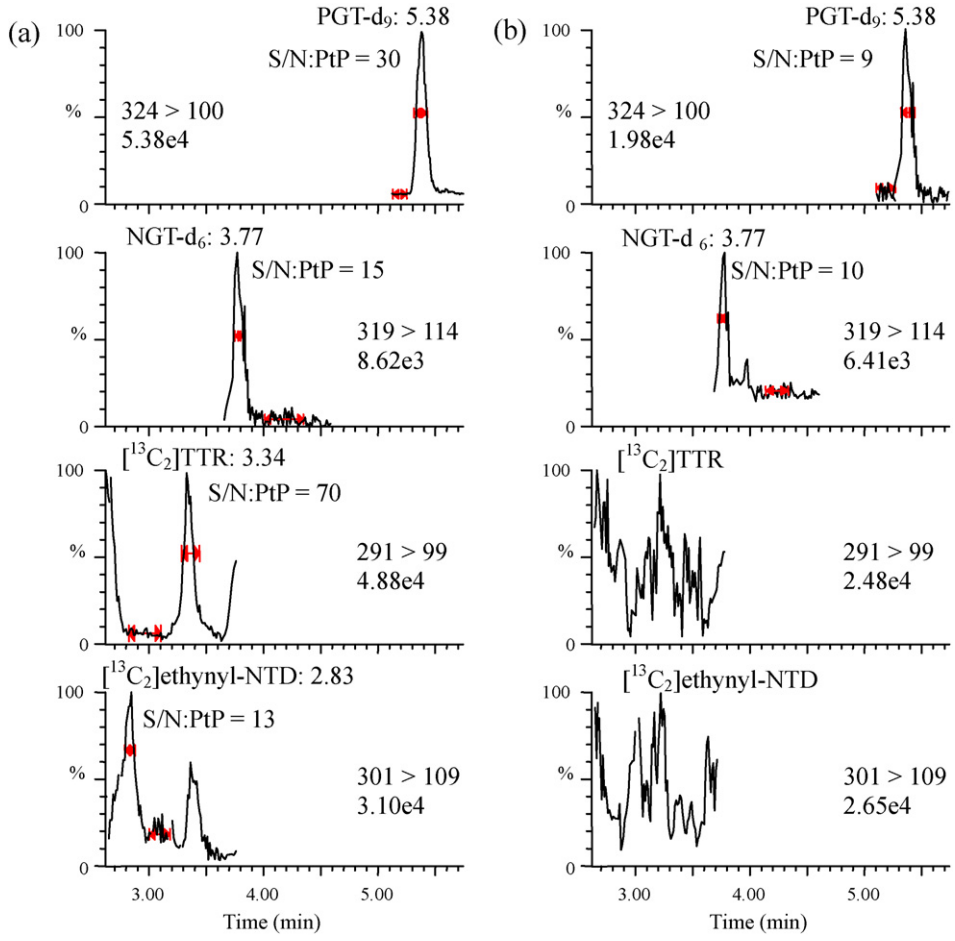

Fig. 3. UPLC–MS/MS MRM chromatograms of four surrogate standards in an influent sample: (a) with cleanup; (b) without cleanup.

Table 3
Mean concentration (ng/L) and matrix spike recoveries of target androgens and progestogens in the wastewater and surface water samples of Japan

Compound	Mean concentration (ng/L)								STP1 influent matrix spike recovery (%)	Surface water site 1 matrix spike recovery (%)
	STP1		STP2		Surface water					
	Influent	Effluent	Influent	Effluent	Site 1	Site 2	Site 3	Site 4		
NAD	–[a]	–	–	–	–	0.25	0.18	–	85	90
TBL	–	–	–	–	–	–	–	–	82	84
NDL	–	–	–	–	–	–	–	–	80	85
ADD	164	5.1	69	5.3	0.32	0.45	0.46	0.28	76	78
NTD	–	–	–	–	–	–	–	–	74	80
17-HPT	–	–	–	–	–	–	–	–	78	81
TTR	15	–	7.9	–	–	–	–	–	84	87
21-HPT	2.1	0.25	2.1	0.25	–	–	–	–	87	79
NGT	–	–	–	–	–	–	–	–	74	75
DPO	1.7	1.4	2.2	1.0	–	–	–	0.15	77	80
MTTR	–	–	–	–	–	–	–	–	82	87
EADR	626	–	1261	–	–	–	–	–	76	80
SZL	–	–	–	–	–	–	–	–	89	92
MHPT	–	0.24	–	1.6	–	–	–	–	80	83
MTA	–	–	–	0.35	–	–	–	–	88	84
MPA	0.21	0.03	2.42	0.42	–	–	–	–	78	81
PGT	10	0.37	3.1	0.31	0.06	0.06	0.09	0.06	94	98
ADR	1102	–	1441	–	–	–	–	–	84	87

[a] Under the method detection limit.

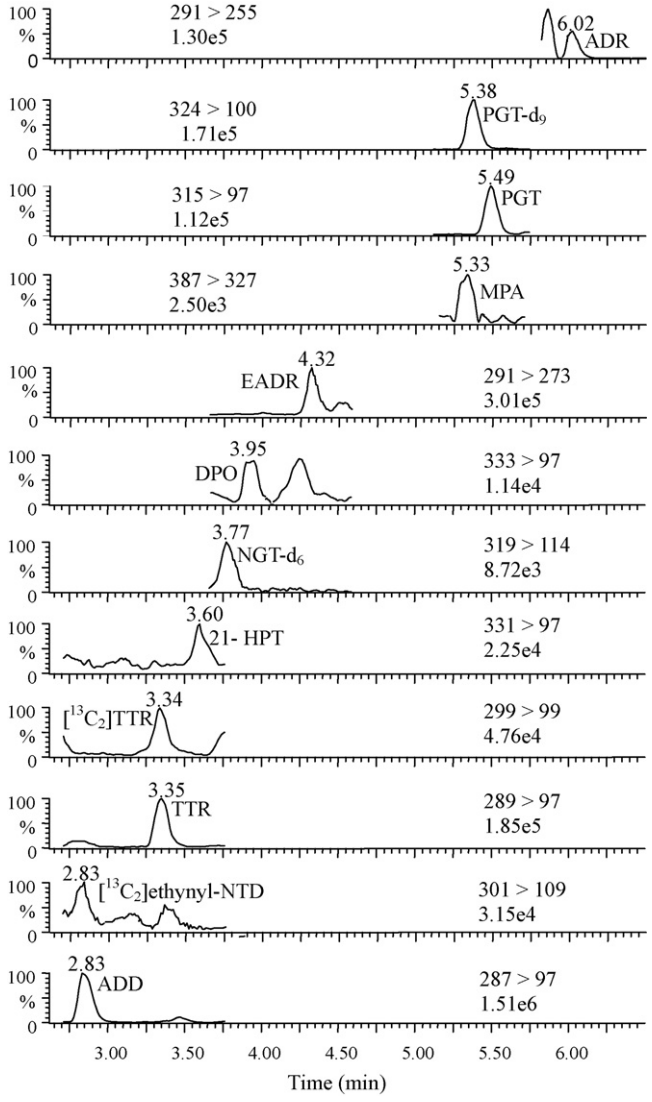

Fig. 4. UPLC–MS/MS MRM chromatograms of androgens and progestogens detected in an influent sample.

4. Conclusion

An UPLC–MS/MS method with a high sensitivity was established for simultaneously analyzing eighteen androgens and progestogens in various water matrices by adapting a cleanup on a silica cartridge. The developed method provided a tool to obtain the simultaneous occurrence data of eighteen androgens and progestogens in environmental waters, which permitted the assessment of their risk to environmental organisms.

Acknowledgements

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