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Simultaneous determination of estrogenic and androgenic hormones in water by isotope dilution gas chromatography–tandem mass spectrometry

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ABSTRACT

A rapid gas chromatography–tandem mass spectrometry (GC–MS/MS) analytical method was developed for the simultaneous analysis of 7 estrogenic hormones (17α-estradiol, 17β-estradiol, estrone, mestranol, 17α-ethynylestradiol, levonorgestrel, estriol) and 5 androgenic hormones (testosterone, androsterone, etiocholanolone, dihydrotestosterone, androstenedione) in aqueous matrices. This method is unique in its inclusion of all 12 of these estrogens and androgens and is of particular value due to its very short chromatographic run time of 15 min. The use of isotope dilution for all analytes ensures the accurate quantification, accounting for analytical variabilities that may be introduced during sample processing and instrumental analysis. Direct isotopically labelled analogues were used for 8 of the 12 hormones and satisfactory isotope standards were identified for the remaining 4 hormones. Method detection levels (MDLs) were determined to describe analyte concentrations sufficient to provide a signal with 99% certainty of detection. The established MDLs for most analytes were 1–5 ng L^{-1} in a variety of aqueous matrices. However, slightly higher MDLs were observed for etiocholanolone, androstenedione, testosterone, levonorgestrel and dihydrotestosterone in some aqueous matrices. Sample matrices were observed to have only a minor impact on MDLs and the method validation confirmed satisfactory method stability over intra-day and inter-day analyses of surface water and tertiary treated effluent samples.

1. Introduction

Estrogenic and androgenic steroid hormones are environmental contaminants of increasing regulatory concern and attention. These include both natural and synthetic substances used for a variety of applications. Some steroidal hormones are used in medicine as contraceptives or in agriculture as growth promoters of meat-producing animals [1–3]. Natural and synthetic steroidal hormones are excreted by humans and animals and can be transferred to surface water by discharging treated municipal wastewaters [4,5] or through run-off from agricultural operations [6,7]. The application of digested municipal sewage sludge to agricultural fields may also be an important pathway for the transfer of steroidal hormones to soil and groundwater [8]. Estrogenic steroids have been reported in treated sewage effluents in many countries across Asia, Europe, Australia and North America [8–15]. Although they have not been subjected to the same degree of scrutiny, androgenic steroid hormones have also been reported in municipal wastewaters in a few studies [16–18].

The main constituent of the estrogenic contraceptive drug, 17α-ethynylestradiol, has been shown to result in localised extinction of some fish species due to reproductive disruption at concentrations of 5–6 ng L^{-1} [19]. The androgenic hormone testosterone can elicit pheromonal responses in fish at nanogram per litre concentrations [20]. Reported impacts of steroidal hormones to aquatic species include behaviour changes [21], morphological abnormalities [22–26], increased occurrence of hermaphrodite organisms and thus reduced reproductive success of fish [21–25,27–31]. Due to the widespread observations of these impacts and ongoing global concern, new analytical developments leading to improved sensitivity, faster analysis times and greater capability for simultaneous analysis of a large number of analytes in water are warranted.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been reported for the analysis of a range of steroidal estrogens and androgens [18,32–37]. While this has proved highly sensitive for the analysis of relatively clean

Abbreviations: BSTFA (99%) + TCMS (1%), N,O-bis(trimethylsilyl) trifluoroacetamide (99%) + trimethylchlorosilane (1%); EI, eleactron ionisation; MW, molecular weight; MRM, multiple reaction monitoring; MDL, method detection level; MBR, membrane bioreactor; HLB, hydrophilic lipophilic balance; SPE, solid phase extraction; Q, quadrupole.

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environmental waters, ion suppression, leading to marked losses of sensitivity can be a significant problem for more complex matrices such as wastewaters [37]. This problem has been particularly observed for androgenic steroids [36].

Gas chromatography–mass spectrometry (GC–MS) has been a preferred technique for determination of steroidal hormones as it is generally able to achieve improved detection limits in more complex matrices [38–50]. Better sensitivity has been achieved by GC coupled with tandem mass spectrometry (GC–MS/MS). A number of GC–MS/MS methods have been developed for the analysis of estrogenic steroids in biological and environmental samples [51–56] and a few GC–MS/MS methods have been developed for the analysis of a wider range of steroidal hormones including a few androgens [16,57]. However, to the best of the author's knowledge, no published methods are currently available for the simultaneous determination of all 7 estrogens and 5 androgens as presented in the current method. Furthermore, the previously published GC–MS/MS methods that have included simultaneous analysis of both androgenic and estrogenic hormones have not incorporated isotope dilution for accurate quantification accounting for extraction losses and potential matrix effects [16,57].

In order to overcome the above limitations, we have developed a simple, reliable and sensitive analytical method for the simultaneous determination of the most common 7 steroidal estrogens and 5 androgens in aqueous environmental matrices. Water samples are extracted by solid phase extraction (SPE) followed by GC–MS/MS analysis using isotope dilution. All the analytes can be monitored in a single GC–MS/MS run with a rapid run time of 15 min.

2. Materials and methods

2.1. Materials and reagents

17α-Estradiol, 17β-estradiol, estrone, estriol, 17α-ethynylestradiol, levonorgestrel, mestranol, testosterone, etiocholanolone, androstenedione, androsterone, dihydrotestosterone, pyridine and 99% N,O-bis(trimethylsilyl)trifluoro-acetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (all analytical grade), Whatman glass fibre filters and filtering system were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). D3-estriol, D3-dihydrotestosterone, D2-testosterone, D4-17α-ethynylestradiol, D4-estrone, D4-17β-estradiol, D2-etiocholanolone were purchased from CDN isotopes Inc., Canada, and D3-androstenedione was purchased from National Measurement Institute, Australia.

Acetonitrile and methanol (anhydrous spectroscopy grade) were purchased from Ajax Finechem (Tarron Point, NSW, Australia). Ultrapure water was produced using a Driec-Q filtering system from Millipore (North Ryde, NSW, Australia). Kimble culture tubes (13 mm I.D. × 100 mm) and a Thermo Speedvac concentrator (Model No. SPD121P) were purchased from Biolab (Clayton, Vic, Australia). Oasis hydrophilic lipophilic balance (HLB) solid phase extraction cartridges (6 mL, 500 mg) were purchased from Waters (Rydalmere, NSW, Australia).

Stock standard solutions of steroidal hormones and isotope labelled steroidal hormones were initially prepared in acetonitrile (500 mg L^{-1}, 20 mL) in amber vials and then further serial diluted with acetonitrile to obtain working standard solutions of lower concentrations. All standard solutions were stored at −18 °C and prepared freshly every three months. Working solutions of steroidal hormones and isotope labelled steroidal hormones at lower concentrations were stored at 4 °C and freshly prepared from concentrated stock standards monthly. Chemical structures of target analytes and their isotope labelled standards used in this study are presented in Table 1.

2.2. Sample collection

All samples were collected in 500 mL amber glass bottles. Ultrapure water was produced using a Driec-Q filtering system from Millipore. Drinking water was collected from a regular potable water tap at UNSW. Membrane Bioreactor (MBR) effluent was the effluent produced by a laboratory-scale MBR treating a synthetic feed solution. The design characteristics, operational parameters and synthetic feed solution of this MBR have been previously described [58]. Surface water was collected from a pond in a large municipal park in Sydney. Tertiary treated effluent was a disinfected final effluent from a municipal wastewater treatment plant in western Sydney. The dissolved organic carbon (DOC) and total suspended solids (TSS) of each of these water matrices are presented in Table 2.

Samples were spiked with stock solutions of all analytes for method recovery and detection level determination. The target concentrations of analytes were dependent on the specific experiments as described in the method validation studies (Section 2.7) below. All samples were then further spiked with isotopically labelled standards for accurate isotope dilution quantification. The target concentrations of the isotope standards were selected to be within an order of magnitude of the spiked analyte concentrations.

Spiked ultrapure water, drinking water and synthetic MBR effluents were extracted without any further treatment or processing. Surface water samples and tertiary treated effluent samples were filtered by 0.75 μm Whatman filter paper prior to extraction. All samples were extracted within 24 h of collection and spiking.

2.3. Solid phase extraction (SPE)

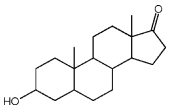
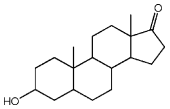
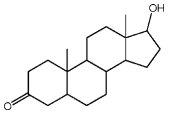
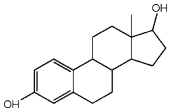
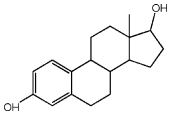
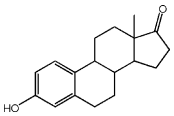
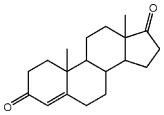
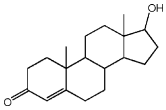
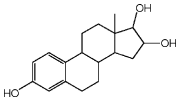
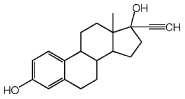
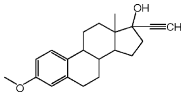
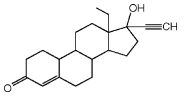
The Oasis HLB SPE cartridges were pre-conditioned prior to extraction with methanol (5 mL), followed by ultrapure water (5 mL). SPE cartridges were loaded by drawing through 500 mL of the aqueous samples under vacuum, maintaining a consistent loading flow rate of less than 5 mL min^{-1}. The SPE cartridges were rinsed with 10 mL of ultrapure water before drying by passing through a flow of nitrogen gas until visibly dry (approximately 1 h). If required, dried cartridges were stored at −18 °C prior to elution and quantitative analysis. Analytes were eluted from the cartridges with methanol (2× 5 mL) into Kimble culture tubes. The extracts were centrifugally evaporated under vacuum at 35 °C using a Thermo Speedvac (Biolab) concentrator. The concentrator was set to an 'auto vacuum' run, with a final pressure of 0.5 Torr. This evaporation process took approximately 1–4 h, depending on the number of samples and the types of matrices (a maximum of 32 samples can be dried in a single batch). The evaporated samples were reconstituted with anhydrous acetonitrile (1 mL) and transferred to amber GC autosampler vials and dried under a gentle nitrogen stream until visibly dry (approximately 3–15 min depending on the types of matrices).

2.4. Trimethylsilyl derivatisation

In preparation for GC–MS/MS analysis, all samples underwent chemical derivatisation. 50 μL of BSTFA (99%)–TCMS (1%), 50 μL of pyridine and 400 μL of acetonitrile (anhydrous grade) were added to the dried samples, then the vials were sealed and heated at 60 °C for 30 min. The derivatised samples were then allowed to cool to room temperature.

It should be noted that this derivatisation process is sensitive to the presence of any moisture. Accordingly, it is important to ensure that the samples are fully dried (as described in the previous section) before the addition of the derivatising reagents and anhydrous acetonitrile. Furthermore, the smallest commercially available bottles of pyridine (100 mL) and anhydrous acetonitrile (100 mL) were

Table 1
Chemical structures of target analytes and their corresponding isotope labelled standards in this study.

Target analytes (corresponding isotope labelled standards)	MW of target analytes (MW of corresponding isotope labelled standards)	Structure of target analytes
Androsterone (16,16-D2-etiocholanolone)	290.4 (292.5)	
Etiocholanolone (16,16-D2-etiocholanolone)	290.4 (292.5)	
Dihydrotestosterone (16,16,17-D3-dihydrotestosterone)	290.4 (293.5)	
17α-Estradiol (2,4,16,16-D4-17β-estradiol)	272.4 (276.4)	
17β-Estradiol (2,4,16,16-D4-17β-estradiol)	272.4 (276.4)	
Estrone (2,4,16,16-D4-estrone)	270.4 (274.4)	
Androstenedione (19,19,19-D3-androstenedione)	286.4 (289.4)	
Testosterone (1,2-D2-testosterone)	288.4 (290.4)	
Estriol (2,4,17-D3-estriol)	288.4 (291.4)	
17α-Ethynylestradiol (2,4,16,16-D4-17α-ethynylestradiol)	296.4 (300.4)	
Mestranol (2,4,16,16-D4-17α-ethynylestradiol)	310.4 (300.4)	
Levonorgestrel (2,4,16,16-D4-17α-ethynylestradiol)	312.5 (300.4)	

used to avoid long storage times of these moderately hygroscopic solvents. Similarly, the mixed derivatising reagent was purchased in 1 mL packs and used only on the same day that they were opened.

Table 2
DOC and TSS of water matrices used for method validation.

	DOC ($mg\,L^{-1}$)	TSS ($mg\,L^{-1}$)
Ultrapure water	0.1	N/A
Drinking water	2	N/A
MBR effluent	8	N/A
Surface water	16	14
Tertiary treated effluent	15	3

N/A: not applicable.

2.5. Gas chromatography–tandem mass spectrometry

Samples were analysed on an Agilent 7890A gas chromatograph (GC) coupled with an Agilent 7000B triple quadrupole mass spectrometer (MS/MS). The GC injection port was operated in splitless mode. The inlet temperature and the GC/MS interface temperature were maintained at 250 °C. An injection volume of 1 μL was used. The inlet was used in splitless mode with a purge time of 1.5 min. Analytes were separated on an Agilent HP5-MS (30 m × 250 μm × 0.25 μm) column using a $0.8\,mL\,min^{-1}$ helium flow. The GC oven temperature was initiated at 130 °C and held for 0.5 min, then increased by $40\,^{\circ}C\,min^{-1}$ to 240 °C, and increased by $5\,^{\circ}C\,min^{-1}$ to 280 °C and held at 280 °C for 3.75 min. The total run time was 15 min.

Mass spectrometric ionisation was undertaken in electron ionisation (EI) mode with an EI voltage of 70 eV and a source temperature of 280 °C. The triple quadrupole MS detector was operated in multiple reaction monitoring (MRM) mode with the gain set to 100 for all analytes. In order to identify the most suitable transitions for MRM, analytical standards were initially analysed in scan mode to identify suitable precursor ions in MS1 with a scan range of m/z 30 to m/z $M+10$ (where M is the derivatised mass of the compound of interest). Fragmentation of the precursor ions in the collision cell was assessed by performing a product ion scan using the same mass range and scan time. All samples were run with a solvent delay of 5 min and the analytes were separated into 3 discrete time segments for MRM aquisition with dwell times ranging from 3 to 25 ms, depending on the time segment, to achieve 10–20 cycles across each peak for good quantification. All ions were monitored at wide resolution (1.2 amu at half height).

The ion transitions monitored for all analytes and isotope standards, as well as the specific dwell times and collision energies for the method are presented in Table 3. The first MRM transition shown for each molecule was used for quantification, while the second transition shown was monitored only for confirmation of molecular identification. A chromatogram showing quantifier peaks of 12 analytes in tertiary treated effluent matrix at a spiking concentration of 10 ng L^{-1} is presented in Fig. 1.

2.6. Identification and quantification

As described in the previous section, two MRM transitions of a single precursor ion were monitored for each target compound. Analysis of the acquired data was undertaken using Agilent MassHunter software. The confirmed identification of a target compound was only established once the analysis met all of the identification criteria. These included the observed presence of the two expected transitions at the same retention time, the area ratio of two transitions within a range of 20% variability with respect to the mean area ratio of all calibration solutions, and a consistent analyte-surrogate relative retention time as that of calibration solutions with relative standard deviation of less than 0.1 min.

2.7. Method validation studies

Isotope labelled compounds were used as surrogate standards to correct for matrix effects, SPE recovery variabilities and instrumental variations for the steroid analytes. Direct analogue isotopic standards were used for etiocholanolone, dihydrotestosterone, 17β-estradiol, estrone, androstenedione, testosterone, estriol and 17α-ethynylestradiol. However, for four of the target analytes, alternative isotope standards were used based on their structural similarity and confirmed suitability (see Section 3.1). Accordingly, D2-etiocholanolone was selected for its stereoisomer androsterone, and D4-17β-estradiol was selected for its stereoisomer 17α-estradiol, and D4-17α-ethynylestradiol was selected as the isotopic standard for mestranol and levonorgestrel. Method recoveries of the target analytes were validated in a variety of matrices including ultrapure water, drinking water, synthetic MBR effluent, natural surface water and tertiary treated effluent. The method recoveries of target analytes in various matrices are presented in Table 4.

SPE absolute recoveries were assessed using the spiked ultrapure water, surface water and tertiary treated effluent samples at both a high concentration (100 ng L^{-1}) and a low concentration (10 ng L^{-1} except for dihydrotestosterone, which was spiked at 20 ng L^{-1} since it has an MDL of 15.8 ng L^{-1}). Because the aim was to assess the loss of the target analytes during SPE extraction, the isotope standards (50 ng) were added to the SPE extracts only after the elution step for direct relative comparison to the analytes. The results of this experiment are presented in Table 5.

To assess potential analyte losses occurring specifically during the drying by Speedvac concentrator and reconstitution steps, 3 centrifuge tubes containing 10 mL anhydrous grade methanol were spiked with 100 ng of the target analytes before being vacuum dried for 3 h and reconstituted in anhydrous acetonitrile. The results of this assessment are presented in Table 6. Further potential losses after reconstitution in anhydrous acetonitrile and during drying under nitrogen gas were also assessed with various drying times of 5 min, 30 min and 1 h. The results of these assessments are also presented in Table 6.

Finally, the impact of any potential sample volume-specific effects, such as SPE breakthrough, was assessed by extracting larger sample volumes (1 L, 2 L, 3 L and 4 L) of tertiary treated effluent, each spiked with 20 ng of each analyte, and comparing the recoveries.

MDLs were determined in each of the matrices described above according to Method 1030C from Standard Methods for the Analysis of Water and Wastewater [59]. For each matrix, seven samples of 500 mL were spiked with target analytes at concentrations close to the expected MDLs. The samples were then spiked with isotopic standards, extracted and analysed through all of the above sample processing and data quantification steps. The seven samples were not analysed sequentially, but were divided into two batches and processed independently on different days to better represent day-to-day variability. MDLs were calculated by multiplying the standard deviation of seven replicates by Student's T value of 3.14 (one-side T distribution for six degrees of freedom at the 99% level of confidence). Where the calculated MDLs were greater than the actual spiked concentration of any target analytes, a further seven replicates spiked with higher concentrations were analysed to calculate revised MDLs for those analytes. Alternatively, where the calculated MDLs were 5 or more times smaller than the actual spiked concentrations, a further seven replicates spiked with lower concentrations were analysed to calculate revised MDLs. This procedure was repeated until MDLs of all target analytes were determined with a signal-to-variability ratio within the bounds of the above criteria. Final MDL values are presented in Table 7.

Instrument stability was assessed on an intra-day and inter-day basis by injecting a standard solution containing all analytes (100 ng mL^{-1}) onto the column three times per day over two separate days and comparing the variation in the signal intensity of each analyte standard from these injections. This variation was expressed at the coefficient of variation (C_V) determined as the ratio of the standard deviation (σ) to the mean (μ). The results of this assessment are presented in Table 8. The absolute stability of the whole method for measuring surface water and tertiary treated effluent samples was also assessed by processing three samples of each matrix at various times within a day and three additional samples for each matrix on a different day. The coefficients of variation for these samples are presented in Table 8. Note that the instrument stability calculation does not include correction by isotope dilution, but the method stability calculation does.

Matrix assessment was undertaken by spiking all of the target analytes (and isotopic standards) into extracted and reconstituted surface water and tertiary treated effluent matrix samples. These spiked matrix samples were then derivatised and analysed by the GC–MS/MS. The absolute signal of each analyte was compared to a standard solution (prepared in acetonitrile) of the same concentration in order to calculate a percentage signal enhancement or suppression. The mean values and standard deviations for triplicate samples are presented in Table 9. Note that these experiments did not include correction of measured ion intensities by isotope dilution.

Quantitative determination of the target analytes was undertaken using external calibration principles combined with the

Table 3
Optimal analyte dependent parameters for tandem mass spectrometry.

Segment start time	Analytes and isotope labelled standards	MRM transitions	Retention time (min)	Dwell time (ms)	Optimum collision energy (V)
7.00 min	Androsterone	347.2 → 271.2	8.58	25	6
		347.2 → 175.1		25	8
	Etiocholanolone	347.2 → 271.2	8.70	25	6
		347.2 → 175.1		25	8
	D2-Etiocholanolone	349.2 → 273.3	8.68	25	6
		349.2 → 175.0		25	8
9.20 min	Dihydrotestosterone	347.2 → 213.2	9.70	3	10
		347.2 → 271.2		3	10
	D3-Dihydrotestosterone	350.1 → 215.1	9.67	3	10
		350.1 → 273.2		3	10
	17α-Estradiol	416.0 → 285.1	9.79	3	10
		416.0 → 326.2		3	5
	17β-Estradiol	416.0 → 285.1	10.25	3	10
		416.0 → 326.2		3	5
	D4-17β-Estradiol	420.0 → 287.2	10.23	3	10
		420.0 → 330.3		3	5
	Estrone	342.1 → 257.1	9.82	3	15
		342.1 → 243.9		3	15
	D4-Estrone	346.3 → 261.2	9.79	3	15
		346.3 → 246.2		3	15
	Androstenedione	286.1 → 109.1	10.10	3	5
		286.1 → 124.1		3	5
	D3-Androstenedione	289.3 → 110.0	10.07	3	5
		289.3 → 127.0		3	5
	Testosterone	360.2 → 174.1	10.41	3	11
		360.2 → 162.1		3	11
	D2-Testosterone	362.1 → 176.1	10.40	3	11
		362.1 → 164.1		3	11
	Mestranol	367.0 → 193.2	10.82	3	17
		367.0 → 173.1		3	17
11.15 min	17α-Ethynylestradiol	425.0 → 193.1	11.45	9	20
		425.0 → 231.2		9	20
	D4-17α-Ethynylestradiol	429.1 → 195.1	11.43	9	20
		429.1 → 233.1		9	20
	Levonorgestrel	355.0 → 167.0	12.13	9	20
		355.0 → 193.0		9	20
	Estriol	504.2 → 324.3	12.58	9	11
		504.2 → 386.3		9	9
	D3-Estriol	507.3 → 327.0	12.55	9	11
		507.3 → 389.4		9	9

isotope dilution technique. Calibration curves were comprised of at least 5 points out of nine calibration points for the non-labelled standards (1.0, 2.5, 5.0, 10, 25, 50, 100, 250, 500 ng mL^{-1} in GC autosampler vials). The lowest calibration point used for each analyte was that corresponding to the lowest concentration above the analyte-specific MDL as shown in Table 7). Isotope standards were added to all calibration solutions in a mass equivalent to the mass of isotope standards added to the samples to be analysed.

3. Results and discussion

3.1. Analyte recovery experiments

The calculated method recoveries of the target compounds in ultrapure water, drinking water, synthetic MBR effluent, surface water and tertiary treated effluent matrices are shown in Table 4. It was observed that the use of isotope dilution satisfactorily corrected for any loss during sample processing,

Table 4
Method recoveries of analytes in various water matrices from a spiking concentration of 100 ng L^{-1}, μ ($\pm\sigma$) %.

Analytes	Method recoveries				
	Ultrapure water (n = 9)	Drinking water (n = 9)	MBR effluent (n = 9)	Surface water (n = 9)	Tertiary treated effluent (n = 9)
Androsterone	110 (±10)	104 (±3)	105 (±4)	103 (±6)	114 (±4)
Etiocholanolone	101 (±5)	98 (±3)	97 (±3)	106 (±5)	100 (±7)
Dihydrotestosterone	98 (±5)	97 (±8)	92 (±7)	93 (±7)	95 (±7)
17α-Estradiol	102 (±2)	101 (±2)	102 (±2)	106 (±5)	96 (±4)
Estrone	116 (±5)	100 (±4)	96 (±2)	100 (±4)	96 (±4)
Androstenedione	104 (±3)	105 (±3)	103 (±3)	105 (±7)	104 (±7)
17β-Estradiol	100 (±2)	98 (±2)	94 (±3)	98 (±7)	98 (±6)
Testosterone	101 (±2)	100 (±4)	100 (±3)	104 (±4)	106 (±7)
Mestranol	90 (±15)	90 (±4)	84 (±2)	80 (±10)	86 (±10)
17α-Ethynylestradiol	112 (±5)	88 (±4)	83 (±2)	81 (±5)	90 (±3)
Levonorgestrel	100 (±15)	100 (±8)	99 (±7)	107 (±7)	120 (±10)
Estriol	101 (±3)	92 (±5)	98 (±3)	94 (±5)	96 (±5)

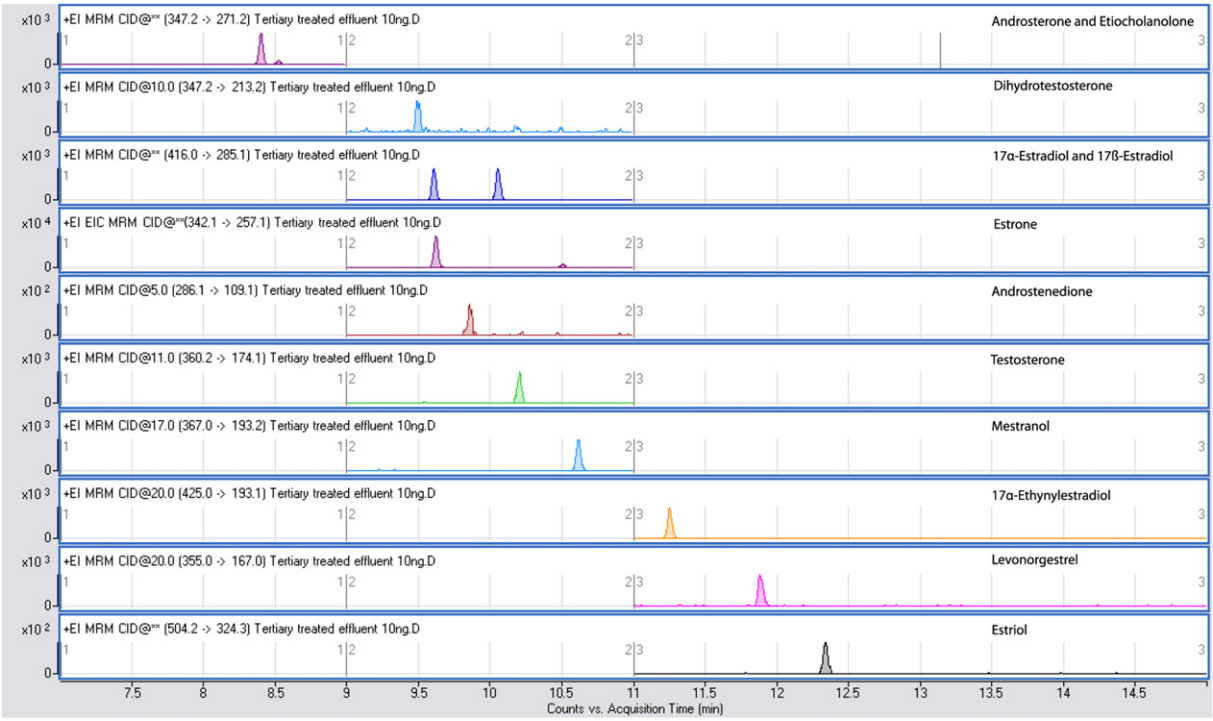


Fig. 1. A chromatogram showing quantifier peaks of 12 analytes in tertiary treated effluent matrix (on column mass = 10 pg).

Table 5
SPE absolute recoveries of analytes from low spiking concentration (10 ng L^{-1}) and high spiking concentration (100 ng L^{-1}), μ ($\pm\sigma$) %.

Analytes	SPE recoveries, 100 ng L^{-1} spiked			SPE recoveries, 10 ng L^{-1} spiked[a]	
	Ultrapure water (n = 3)	Surface water (n = 3)	Tertiary treated effluent (n = 3)	Surface water (n = 3)	Tertiary treated effluent (n = 3)
Androsterone	90 (±3)	101 (±1)	106 (±3)	107 (±3)	102 (±3)
Etiocholanolone	87 (±5)	89 (±7)	102 (±3)	106 (±1)	100 (±3)
Dihydrotestosterone	92 (±7)	92 (±7)	100 (±5)	100 (±3)	104 (±5)
17α-Estradiol	97 (±3)	92 (±7)	93 (±1)	98 (±4)	87 (±5)
Estrone	95 (±2)	95 (±6)	99 (±5)	105 (±4)	104 (±4)
Androstenedione	86 (±7)	92 (±7)	97 (±2)	103 (±7)	109 (±7)
17β-Estradiol	95 (±2)	95 (±5)	92 (±2)	98 (±5)	102 (±4)
Testosterone	95 (±6)	96 (±6)	102 (±6)	96 (±7)	97 (±7)
Mestranol	52 (±6)	95 (±2)	96 (±3)	96 (±2)	97 (±5)
17α-Ethynylestradiol	98 (±2)	92 (±5)	99 (±2)	92 (±5)	98 (±6)
Levonorgestrel	68 (±2)	105 (±7)	109 (±6)	104 (±7)	107 (±7)
Estriol	97 (±3)	98 (±6)	95 (±2)	91 (±7)	93 (±4)

[a] Except for dihydrotestosterone, which was spiked at 20 ng L^{-1} since it has an MDL of 15.8 ng L^{-1}.

Table 6
Recoveries during drying/reconstituting by Speedvac concentrator and drying by nitrogen gas from a spiking concentration of 100 ng L^{-1}, μ ($\pm\sigma$) %.

Analytes	Recoveries during drying and reconstituting by Speedvac concentrator		Recoveries during drying by nitrogen gas	
	Dry 3 h (n = 3)	Dry 5 min (n = 3)	Dry 30 min (n = 3)	Dry 60 min (n = 3)
Androsterone	99 (±2)	97 (±4)	100 (±2)	90 (±5)
Etiocholanolone	96 (±5)	97 (±6)	93 (±5)	90 (±7)
Dihydrotestosterone	105 (±3)	110 (±8)	112 (±2)	104 (±9)
17α-Estradiol	99 (±4)	105 (±3)	108 (±3)	107 (±5)
Estrone	97 (±2)	100 (±4)	110 (±5)	106 (±3)
Androstenedione	100 (±7)	95 (±5)	103 (±9)	105 (±7)
17β-Estradiol	100 (±2)	106 (±4)	104 (±4)	106 (±3)
Testosterone	107 (±9)	106 (±9)	107 (±8)	108 (±6)
Mestranol	99 (±5)	108 (±5)	110 (±3)	102 (±3)
17α-Ethynylestradiol	100 (±4)	107 (±4)	104 (±3)	101 (±5)
Levonorgestrel	104 (±7)	90 (±10)	91 (±11)	89 (±9)
Estriol	101 (±5)	102 (±3)	109 (±5)	105 (±9)

matrix effects and instrument variation leading to accurate quantification in all tested matrices. D2-etiocholanolone and D4-17β-estradiol were confirmed to be suitable isotope standards for the quantification of their stereoisomers androsterone and 17α-estradiol, respectively, with method recoveries in all tested matrices between 96% and 114% (max σ = 10%). Similarly, D4-17α-ethynylestradiol was confirmed to be a reasonable isotopic standard for quantification of mestranol and lev-

Table 7
MDLs of target analytes in various water matrices.

Analytes	MDLs ($ng\,L^{-1}$)				
	Ultrapure water ($n=7$)	MBR effluent ($n=7$)	Drinking water ($n=7$)	Surface water ($n=7$)	Tertiary treated effluent ($n=7$)
Androsterone	1.0	1.0	1.0	1.2	1.4
Etiocholanolone	5.0	5.0	5.0	5.8	6.4
Dihydrotestosterone	8.9	11.3	15.2	15.8	15.0
17α-Estradiol	0.8	0.9	1.2	0.9	1.0
Estrone	0.7	0.7	0.7	0.7	0.8
Androstenedione	5.0	5.0	5.0	5.0	5.5
17β-Estradiol	1.1	1.3	1.2	1.2	1.3
Testosterone	5.0	5.0	5.0	5.0	6.0
Mestranol	1.0	1.3	1.0	1.0	1.2
17α-Ethynylestradiol	1.0	1.0	1.0	1.3	1.2
Levonorgestrel	5.0	6.0	7.5	5.0	7.0
Estriol	2.5	2.5	2.5	2.6	3.0

Note: Injection volume is 1 μL, thus 1 $ng\,L^{-1}$ is equal to 1 pg on column mass.

Table 8
Coefficient of variation $C_v = \sigma/\mu$ for instrument stability and method stability of target analytes in various water matrices.

Analytes	Instrument stability[a]		Method stability[b]			
	Standard 100 $ng\,mL^{-1}$		Surface water 100 $ng\,L^{-1}$		Tertiary treated effluent 100 $ng\,L^{-1}$	
	Intra-day ($n=3$)	Inter-day ($n=6$)	Intra-day ($n=3$)	Inter-day ($n=6$)	Intra-day ($n=3$)	Inter-day ($n=6$)
Androsterone	0.08	0.11	0.06	0.07	0.05	0.07
Etiocholanolone	0.03	0.12	0.04	0.04	0.04	0.04
Dihydrotestosterone	0.08	0.10	0.04	0.07	0.02	0.02
17α-Estradiol	0.02	0.08	0.03	0.02	0.02	0.05
Estrone	0.04	0.10	0.01	0.06	0.01	0.06
Androstenedione	0.10	0.12	0.02	0.07	0.01	0.07
17β-Estradiol	0.02	0.09	0.02	0.04	0.04	0.05
Testosterone	0.06	0.11	0.02	0.05	0.02	0.05
Mestranol	0.05	0.12	0.03	0.06	0.06	0.10
17α-Ethynylestradiol	0.04	0.12	0.01	0.06	0.03	0.04
Levonorgestrel	0.07	0.13	0.05	0.09	0.05	0.10
Estriol	0.05	0.10	0.01	0.05	0.01	0.05

[a] Instrument stability not corrected by isotope dilution.
[b] Method stability includes correction by isotope dilution.

Table 9
Signal enhancement/suppression in surface water and tertiary treated effluent matrices from a spiking concentration of 20 $ng\,L^{-1}$, μ ($\pm\sigma$) %.

	Surface water matrix ($n=3$)	Tertiary treated effluent matrix ($n=3$)
Androsterone	−4 (±9)	−13 (±7)
Etiocholanolone	−18 (±10)	−25 (±8)
Dihydrotestosterone	+9 (±9)	+15 (±2)
17α-Estradiol	−5 (±10)	−8 (±8)
Estrone	−8 (±6)	−1 (±5)
Androstenedione	−10 (±9)	+19 (±10)
17β-Estradiol	−8 (±10)	−7 (±9)
Testosterone	+15 (±11)	+24 (±10)
Mestranol	−3 (±9)	+5 (±9)
17α-Ethynylestradiol	−5 (±7)	+12 (±7)
Levonorgestrel	−9 (±10)	+11 (±10)
Estriol	−1 (±7)	+9 (±9)

onorgestrel with method recoveries from 80% to 120% (max σ = 15%).

The results of SPE absolute recoveries of the target compounds from low concentration (10 $ng\,L^{-1}$) and high concentration (100 $ng\,L^{-1}$) spiking tests are presented in Table 5. In surface water and tertiary treated effluent matrices, the absolute SPE recoveries ranged from 89% to 109% when spiked at 100 $ng\,L^{-1}$ and from 87% to 109% when spiked at 10 $ng\,L^{-1}$. Interestingly, the absolute recoveries from ultrapure water spiked at 100 $ng\,L^{-1}$, were somewhat lower (52–97%) suggesting that dissolved organic carbon in the matrix may enhance the SPE recovery. A possible explanation may be that the organic matrix materials improve the method performance by competing for active adsorption sites on glassware and the GC inlet liner. This would improve quantitative steroid transfer through to the MS detector. Regardless of the cause, these observed matrix differences emphasise the importance of isotope dilution for SPE recovery correction among diverse matrices.

The mean analyte recoveries from spiked methanol samples after drying by the Speedvac concentrator are shown in Table 6. This table also shows the recoveries of the analytes from evaporation of anhydrous acetonitrile samples after evaporation under nitrogen with various drying times (5 min, 30 min and 60 min). The results of these two experiments confirm that negligible losses of all analytes occurred under all of the tested drying conditions.

The results of the recovery experiments from larger sample volumes of tertiary treated effluent (not shown) indicate that recovery efficiencies for all analytes were not detrimentally affected for sam-

ple volumes up to 1 L. This suggests that the MDLs may be driven somewhat lower by the use of 1 L samples instead of 0.5 L samples in some circumstances. However, recoveries of most of the analytes were diminished by up to 50% for sample volumes of 2 L or greater.

3.2. Method detection levels

The MDLs in the different water matrices are presented in Table 7. These results show that in ultrapure water, drinking water, synthetic MBR effluent, surface water and tertiary treated effluent, MDLs typically ranged between 1 and 5 ng L^{-1}. However, slightly higher MDLs were observed for etiocholanolone (up to 6.4 ng L^{-1}), androstenedione (up to 5.5 ng L^{-1}), testosterone (up to 6.0 ng L^{-1}), levonorgestrel (up to 7.5 ng L^{-1}) and dihydrotestosterone (up to 15.8 ng L^{-1}) in some aqueous matrices. Numerous previous studies have reported the presence of estrogenic hormones in effluents of sewage treatment plants at concentrations of 1–70 ng L^{-1} [8,16,54]. Furthermore, estrogenic hormones have been reported at up to 6 ng L^{-1} in impacted surface waters [9]. Much fewer data are available for androgenic hormones, but some have been reported in surface water at concentrations up to 12 ng L^{-1} [18].

Dihydrotestosterone was the least sensitive target compound with generally higher MDLs in ultrapure water (8.9 ng L^{-1}), synthetic MBR effluent (11.3 ng L^{-1}), drinking water (15.2 ng L^{-1}), surface water (15.8 ng L^{-1}) and tertiary treated effluent (15.0 ng L^{-1}). However, these elevated MDLs were the consequence of a decision to base the quantification of this analyte on the most specific (but not most intense) ion transition at m/z 347.2 → m/z 213.2. This decision was made in order to facilitate the clear distinction of dihydrotestosterone from androsterone and etiocholanolone. If required, reduced MDLs for dihydrotestosterone can be achieved by alternatively basing the quantification on the more intense m/z 347.2 → m/z 271.2 transition.

The fact that the MDLs were not significantly reduced from ultrapure water to more complex matrices highlights the robustness of this method against potential impacts of matrix-specific ion suppression during mass spectral analysis.

Some previous studies have quoted lower detection limits for some of the analytes presented in this paper. While the approach taken to determine these detection limits has been variable (and often not explicitly stated), the most common procedure has been to identify an analyte concentration for which a signal-to-noise ratio (S/N) of 3 can be obtained. The concentration obtained by this approach is most correctly termed the 'lower level of detection' (LLD) or the 'level of detection' (LOD) [59]. This approach is intended to set the probability of both false positives and false negatives at 5%. However, the LLD method is not well suited to GC–MS/MS analysis since it is commonly not possible to observe any 'noise' (for example, see Fig. 1). A more robust (but somewhat more conservative) approach for defining detection limits is adopted in this paper, as has been referred to as the 'method detection level' (MDL). The MDL is used to describe the analyte concentration that, when processed through the complete method, produces a signal with a 99% probability that it is different from the blank [59].

The better sensitivity of the estrogens compared to the androgens is assumed to be largely due to differences in EI fragmentation at 70 eV. Fragmentation of estrogens generally resulted in the production of around 6–10 highly stable ion fragments (as observed in full scan mass spectra, not shown). However, the androgens were typically fragmented into a much larger number of ion fragments, thus the overall signal was distributed (or diluted) between a larger number of m/z values. The stable ion formation for many of the estrogens (with the exception of mestranol) may be partially due to the TMS-derivatised phenol group, which the androgens lack.

3.3. Instrument stability, matrix effects and calibration range

The results of instrument and method stability assessments are presented in Table 8. The coefficients of variability ($C_v = \sigma/\mu$) for instrument variability on an intra-day basis ranged from 0.02 to 0.10. Slightly greater coefficients of variability for instrument variability were observed on an inter-day basis, from 0.08 to 0.13. However, the coefficients of variability for the full method analysis of spiked surface water and tertiary effluent samples, on both an intra-day and inter-day basis were observably lower. These varied from 0.01 to 0.07 for analytes with direct isotope labelled analogue correction and up to 0.10 for analytes with alternative isotope labelled analogue correction. This observation emphasises the importance of the isotope dilution process to ensure a high level of analytical reproducibility.

The results of the signal enhancement/suppression assessment in surface water and tertiary treated effluent matrices are presented in Table 9. These data represent the means and standard deviations of three samples assessed in each of the two matrices. Some degree of signal suppression may be evident for a few analytes (e.g. etiocholanolone) and enhancement for others (e.g. testosterone). However, these results reveal a high degree of variability between samples, thus obscuring any real trends. This variability again reinforces the importance of isotope dilution for accurate quantification in real sample matrices.

Blank (unspiked) matrix samples were run to assess background concentrations of the analytes in ultrapure water, drinking water, synthetic MBR effluent, surface water and tertiary treated effluent. The only observed analyte in these matrix samples was estrone, which was measured in tertiary treated effluent at a concentration of 1 ng L^{-1}. Accordingly, all validation experiments on this matrix were calculated after correcting for a background concentration of 1 ng L^{-1} estrone.

The linear calibration range for the target compounds was determined to be from their identified MDLs to 500 ng L^{-1}, thus the upper quantification limit is 500 ng L^{-1} for all analytes. The calibration points for each of the analytes were fitted to linear regressions and the calibration curve regression correlation coefficients were always at least 0.99 for all sample batches.

4. Conclusion

An analytical method was developed for the simultaneous analysis of 12 natural and synthetic hormones in aqueous matrices. No previous GC–MS method is known that encompasses this full range of estrogenic and androgenic analytes. Furthermore, the use of GC–MS/MS has enabled unambiguous identification and non-interfering quantification of closely eluting chromatographic peaks in a very short analysis time of only 15 min.

The use of isotope dilution for all analytes ensures the accurate quantification, accounting for analytical variabilities that may be introduced during sampling, extraction, derivatisation, chromatography, ionisation or mass spectrometric detection. Direct isotopically labelled analogues were used for 8 of the 12 hormones. However, satisfactory isotope standards were determined for the remaining 4 hormones, based on structural similarity and observed method recoveries of 80–120% in all sample matrices.

The established MDLs for most analytes were 1–5 ng L^{-1} in a variety of aqueous matrices. However, slightly higher MDLs were observed for etiocholanolone, androstenedione, testosterone, lev-

onorgestrel and dihydrotestosterone in some aqueous matrices. Sample matrices were observed to have only a minor impact on MDLs indicating that interferences such as ion suppression, which is a common problem for HPLC–MS (or HPLC–MS/MS) methods, did not have a significant impact on sensitivity for this method. The method validation confirmed very good method stability over intra-day and inter-day analyses.

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