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An on-line solid phase extraction method coupled with UHPLC-MS/MS for the determination of steroid hormone compounds in treated water samples from waste water treatment plants

Rayco Guedes-Alonso, Zoraida Sosa-Ferrera and José Juan Santana-Rodríguez*

An on-line solid phase extraction coupled with ultra-high performance liquid chromatography in tandem with mass spectrometry (SPE-UHPLC-MS/MS) method for the determination of fourteen hormones (four oestrogens, three androgens, four progestogens and three corticosteroids) in waste water samples has been developed. All of the parameters involved in the on-line extraction process have been optimized: type of cartridge, sample volume, loading solvent, solvent of the wash step and the pH of the sample. Moreover, the chromatographic separation and all of the parameters involved in the detection by mass spectrometry have been studied too. The developed method allows for complete analysis (extraction and identification of the analytes) in 14.5 minutes. The method is selective, with satisfactory relative standard deviations (lower than 15% in most cases) and limits of detection and quantification that ranged from 0.5 to 13.2 ng L^{-1} and from 1.66 to 44 ng L^{-1}, respectively. The recoveries were acceptable for most compounds for effluent samples from different waste water treatment plants (between 50 and 90%). The proposed method has been applied to study effluent samples from three waste water treatment plants from Gran Canaria (Spain). Four steroid hormones of different families have been detected at concentrations ranging from 3.1 to 52.8 ng L^{-1}.

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1. Introduction

In recent years, endocrine disrupting compounds (EDCs) have garnered increasing attention from the international community. Changes in aquatic biota, such as hermaphroditism, feminization, inhibition of locomotion and aggressive behaviour or changes in fertility or vitellogenin, are produced by these types of emerging pollutants, which have been discussed in several studies.[1–4] Among the EDCs, we can consider steroid hormones as a wide group that can be divided into four subgroups: oestrogens, androgens, progestogens and corticosteroids. Oestrogens, such as 17β-estradiol (E2), estrone (E1) and oestriol (E3), are female hormones that are essential to the menstrual cycle of women. Natural and synthetic oestrogens are used in both human and veterinary medicine with the main medical application being birth control. The most used synthetic oestrogen for birth control is 17α-ethinyloestradiol (EE). Progestogens, also called gestagens, are characterized by their basic 21-carbon skeleton and their main function is to maintain the pregnancy, although they are expressed in several phases of the menstrual cycle. Consequently, progestogens are also used as hormonal contraceptives that can be combined with oestrogens. In the last decade, the consumption of oestrogens, with and without combination with progestogens, has greatly increased. In fact, currently, 100 million women are active users of combined hormonal contraceptives worldwide.[5] Alternatively, androgens are frequently used by some sportsmen to increase their strength, mass and muscular size. Nevertheless, the doses are higher than the doses used in hormone replacement therapies; in consequence, serious side effects can appear, such as testicular atrophy, sterility, gynecomastia in males and ovulation inhibition, hirsutism, alopecia and acne in females.[6] Finally, corticosteroids are synthesized in the adrenal cortex of vertebrates and are involved in many physiological processes. Corticosteroids are divided into mineralocorticoids and glucocorticoids and these substances can be artificially synthesized for therapeutic applications due to their anti-inflammatory properties and immunosuppressive effects on metabolism. Corticosteroids are illegal in the EU for fattening purposes as legislated in the 96/22/EC directive.[7]

A significant quantity of consumed hormones exit organisms through excretions.[8,9] For this reason, most publications agree that waste water treatment plants (WWTPs) are the principal sources of EDC release into the environment.[10] The presence of hormone compounds in the effluents of WWTPs is due to their incomplete degradation by treatment processes, which produces an alarming contamination in aquatic

Departamento de Química, Universidad de Las Palmas de Gran Canaria, 35017, Las Palmas de Gran Canaria, Spain. E-mail: josejuan.santana@ulpgc.es

Table 1 List of hormone compounds, surrogate standards, pK_a values, and retention times

Type of hormone	Abbreviation	Compound	Surrogate standard	pK_a[35]	t_R (min)
Oestrogens	E3	Estriol	Estrone D2	10.3	6.50
	E2	17β-estradiol		10.3	7.07
	E1	Estrone		10.3	7.07
	DES	Diethylstilbestrol		10.2	7.08
Progestogens	NORET	Norethisterone	Progesterone D9	13.1	7.05
	NOR	Norgestrel		13.1	7.20
	MGA	Megestrol acetate		—	7.32
	PRO	Progesterone		—	7.40
Androgens	BOL	Boldenone	Testosterone D3	15.1	7.00
	NAN	Nandrolone		15.1	7.05
	TES	Testosterone		15.1	7.15
Corticosteroids	PRED	Prednisone	Progesterone D9	12.4	6.60
	COR	Cortisone		12.4	6.63
	PREDNL	Prednisolone		12.5	6.73

environments.[11–13] The compound concentrations found in the environment are in the range of ng L^{-1} [12,14,15] because of the low doses of these drugs, their catabolism by humans and most of them degrade in WWTPs.

Because of the low concentration of steroid hormones in the environment, it is necessary to develop sensitive methods for extraction, preconcentration and identification of hormones in water samples. Solid phase extraction (SPE) is a widespread method of extraction used to isolate and preconcentrate emerging pollutants from aqueous matrices.[16–18] Some authors have reported extraction of oestrogens, androgens, progestogens and corticosteroids from WWTP samples using this method in the last decade.[18–21] The separation and identification techniques used more often in recent years have been liquid chromatography with mass spectrometry detection (LC-MS)[22] and liquid chromatography in tandem with mass spectrometry detection (LC-MS/MS).[19,20,23,24] These techniques allow the identification of hormones without a derivatization step, which is needed when using GC-MS.[25–27] On-line SPE methods have been developed in recent years and present advantages over off-line SPE methods, such as lower sample handling and analysis time. On-line SPE coupled to HPLC-MS/MS and UHPLC-MS/MS provides a highly sensitive and specific method for steroid hormone detection in water samples.[20,24,28]

In this study, an on-line SPE process coupled with liquid chromatography in tandem with mass spectrometry detection system has been developed for the determination of fourteen steroid hormones belonging to four subgroups (Table 1). All of the conditions involved in the extraction, separation and identification processes have been optimized using the effluent

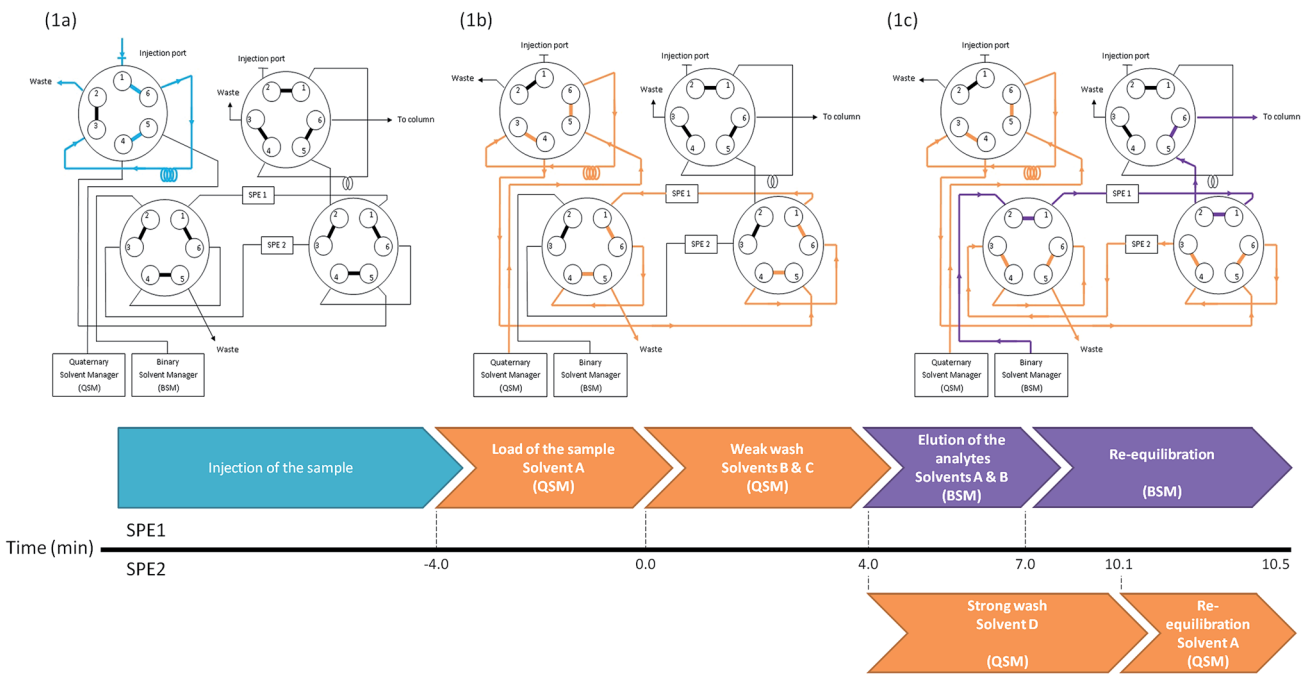

Fig. 1 Positions of the valves and solvents in different on-line SPE events.

Table 2 Gradient used in Binary and Quaternary solvent Managers[a]

	Binary solvent manager			Quaternary solvent manager					
				Flow (mL min^{-1})					
Time (min)	Flow (mL min^{-1})	A (%)	B (%)	OASIS HLB	XBridge C_{18}	A2 (%)	B2 (%)	C (%)	D (%)
0.0	0.30	80	20	2.00	2.00	100	0	0	0
3.8	0.30	80	20	2.00	0.01	0	100	0	0
4.1	0.30	80	20	2.00	0.01	0	100	0	0
7.0	0.30	0	100	2.00	2.00	0	0	0	100
8.0	0.30	0	100	2.00	2.00	100	0	0	0
10.5	0.30	80	20	2.00	2.00	100	0	0	0

[a] A: water + 0.1% NH_3, A2: water + 0.05% formic acid, B: methanol, B2: water, C: methanol, D: acetone : hexane : methanol (1 : 1 : 1).

from a tertiary treatment used at a waste water treatment plant (WWTP1). The developed method has been applied to study effluent samples from three WWTPs (WWTP2, WWTP3 and WWTP4) located in Gran Canaria Island (Spain) which use different water treatments. WWTP2 uses a membrane bioreactor for biological treatment and WWTP3 and WWTP4 use the traditional activated sludge treatment.

2. Materials and methods

2.1. Reagents

All of the hormone compounds were purchased from Sigma-Aldrich (Madrid, Spain). The purities of all compounds under study are over 99.0%. Three surrogate standards were used: estrone D2 and progesterone D9 from CDN Isotopes Inc. (Quebec, Canada) and testosterone D3 from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Stock solutions containing 1000 mg L^{-1} of each analyte were prepared by dissolving the compound in methanol and stored in glass-stoppered bottles at −20 °C prior to use. Working aqueous standard solutions were prepared daily. Ultrapure water used in the on-line SPE process was obtained by using a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol, LC-MS methanol, LC-MS water, HPLC-grade *n*-hexane and HPLC-grade acetone, as well as ammonia, ammonium acetate and acetic acid used to adjust the pH of the mobile phases, were obtained from Panreac Química (Barcelona, Spain).

2.2. Sample collection

Water samples were collected from the effluents of four waste water treatment plants located in Gran Canaria in May of 2014 and January of 2015. WWTP1 samples were from the effluent of the tertiary process and were used to optimize the on-line SPE method. WWTP2 has a population equivalent of 7000 and uses

Table 3 Mass spectrometer parameters for the determination of target analytes

Compound	Precursor ion (*m*/*z*)	Cone voltage (ion mode)	Quantification ion, *m*/*z* (collision potential, V)	Confirmation ion, *m*/*z*, (collision potential, V)	Confirmation – Quantification ion ratio
E3	287.2	−65 V (ESI−)	171.0 (37)	145.2 (39)	0.19
PRED	359.3	30 V (ESI+)	147.0 (15)	237.0 (20)	0.25
COR	361.3	30 V (ESI+)	163.0 (25)	121.0 (45)	0.10
PREDNL	361.3	20 V (ESI+)	147.1 (20)	173.1 (25)	0.39
BOL	287.2	30 V (ESI+)	121.0 (28)	135.1 (15)	0.59
NAN	275.2	35 V (ESI+)	109.1 (20)	83.0 (30)	0.53
NORET	299.2	30 V (ESI+)	109.1 (25)	91.0 (40)	0.59
E2	271.2	−65 V (ESI−)	145.1 (40)	183.1 (31)	0.23
E1	269.2	−65 V (ESI−)	145.0 (36)	143.0 (48)	0.22
DES	267.1	−50 V (ESI−)	237.1 (29)	251.1 (25)	0.91
TES	289.2	38 V (ESI+)	97.0 (22)	109.0 (21)	0.80
NOR	313.2	38 V (ESI+)	109.0 (26)	245.1 (18)	0.56
MGA	385.5	30 V (ESI+)	267.3 (15)	224.2 (30)	0.66
PRO	315.3	30 V (ESI+)	97.0 (18)	109.1 (25)	0.86
Deuterated Compound	**Precursor ion (*m*/*z*)**	**Cone voltage (ion mode)**	**Quantification ion, *m*/*z* (collision potential, V)**	**Confirmation ion, *m*/*z* (collision potential, V)**	**Confirmation – quantification ion ratio**
E1-d2	271.2	70 V (ESI−)	147.1 (30)	145.1 (35)	0.12
TES-d3	292.2	35 V (ESI+)	97.1 (25)	109.1 (20)	0.80
PRO-d9	324.3	35 V (ESI+)	100.1 (20)	113.1 (20)	0.56

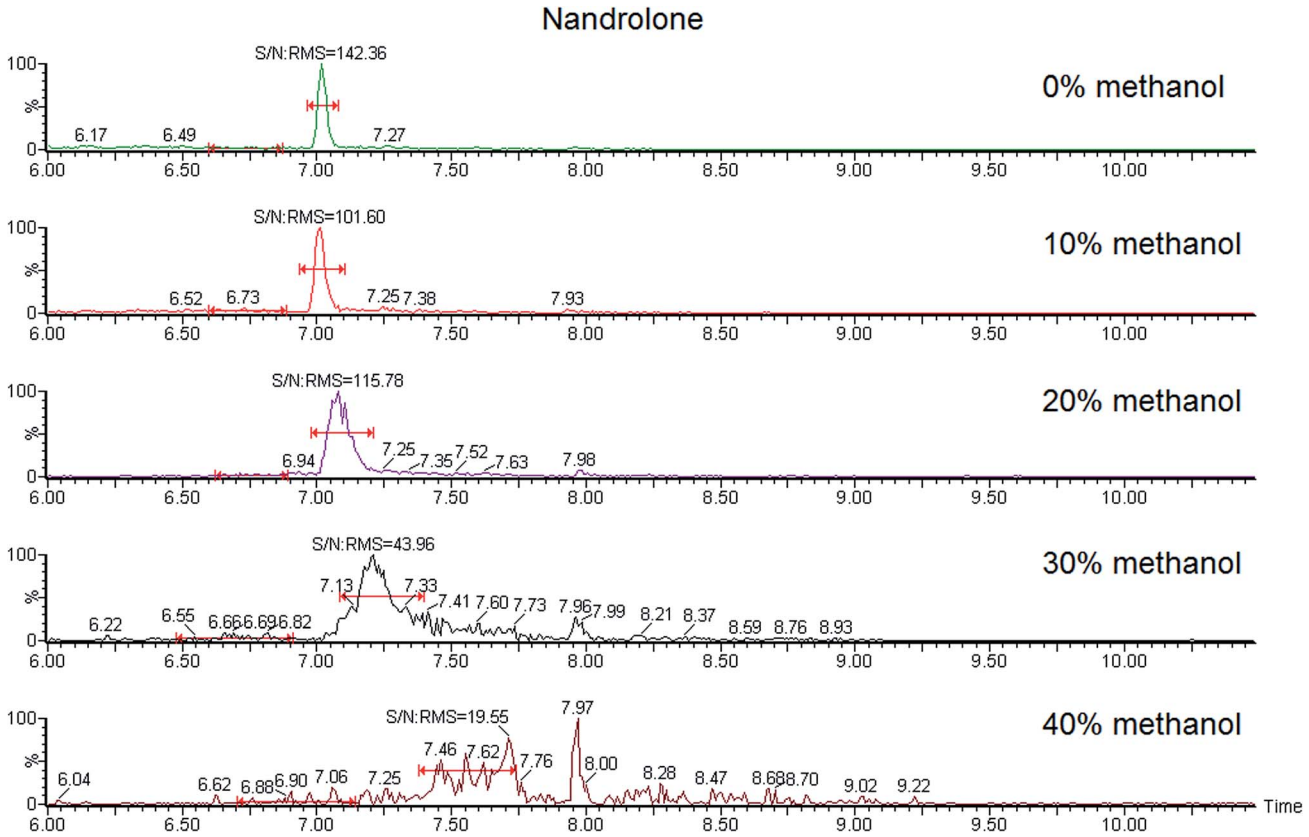

Fig. 2 Effect of methanol of the wash step in the peak detection of nandrolone, including the *S/N* ratio.

a membrane bioreactor treatment system. WWTP3 and WWTP4 treat the water from big urban areas of Gran Canaria (population equivalents of 60 000 and 130 000, respectively) and use an activated sludge treatment. The samples were collected in 2 L amber glass bottles that were rinsed beforehand with methanol and ultrapure water. Samples were acidified to inhibit microbial activity and purified through filtration with fibreglass filters and then with 0.22 μm membrane filters (Millipore, Ireland). The samples were stored in the dark at 4 °C and they were analyzed within 48 hours.

2.3. On-line SPE-UHPLC system

The on-line SPE-UHPLC-MS/MS system used was obtained from Waters (Waters Chromatography, Barcelona, Spain). This system consisted of a binary solvent manager (BSM) pump for the chromatographic separation, a quaternary solvent manager (QSM) pump to perform the extraction process, an autosampler capable of injecting volumes up to 5000 μL per injection, a column manager and a triple quadrupole detector (TQD). Solid phase extraction was performed using OASIS HLB (2.1 × 30 mm, 20 μm) and XBridge C_{18} (2.1 × 30 mm, 10 μm) SPE columns (Waters Chromatography, Barcelona, Spain) followed by elution of the analytes with the chromatographic mobile phase and finally, separation was achieved in the analytical column placed in the column manager.

A scheme of the on-line SPE process is shown in Fig. 1. First, the autosampler injects a volume of up to 5000 μL into valve 2 and the sample is placed in the loop (Fig. 1a). Next is the loading phase (solvent A of the QSM) where the sample is loaded into the SPE column 1. After loading, solvents B and C of the QSM perform the wash step to eliminate interferents in the sample (Fig. 1b). After the wash step, a change in the valves allows for column 2 to be strongly washed with a mixture of organic solvents (solvent D of the QSM) while SPE column 1 is eluted with the chromatographic mobile phase of the binary solvent manager (BSM) (Fig. 1c). After the strong wash and during the chromatographic separation, SPE column 2 is conditioned and equilibrated with the load phase (solvent A of the QSM) to prepare it for the next extraction.

In this system the solvent pumps have different purposes. The quaternary solvent manager (QSM) is used to load the sample into the SPE column, a weak wash of the SPE column to eliminate interferents and to strongly wash the SPE columns to eliminate any analyte retention. The binary solvent manager serves for elution of the analytes to the separation column and chromatographic analyses.

2.4. Chromatographic and mass spectrometry conditions

2.4.1. Chromatographic separation. The analytical column used was a 50 mm × 2.1 mm, ACQUITY UHPLC BEH Waters C_{18} column with a particle size of 1.7 μm (Waters Chromatography, Barcelona, Spain) operating at a temperature of 30 °C. The injection volume was 2 mL of the sample and the mobile phases were water, with 0.1% v/v ammonia (A) and methanol (B). The

Table 4 Analytical parameters of target analytes for every sample studied

		Effluent WWTP1				Effluent WWTP2			
		100 ng L^{-1}		500 ng L^{-1}		100 ng L^{-1}		500 ng L^{-1}	
Compound	LOD[a] (ng L^{-1})	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$
Diethylstilbestrol	13.2	44.3	7.3	42.3	14.7	44.3	14.6	51.9	4.7
17β-estradiol	8.5	88.8	26.4	104.0	7.0	126.7	14.6	112.8	6.2
Estrone	4.1	75.1	15.1	81.6	8.8	75.5	15.8	82.6	5.3
Estriol	4.5	76.8	5.2	69.7	17.1	58.6	16.9	78.5	11.0
Norgestrel	1.6	34.5	8.6	36.7	11.6	42.5	8.1	48.4	6.1
Testosterone	1.0	53.1	6.9	52.3	3.7	69.7	6.3	74.4	2.8
Megestrol acet.	1.2	138.7	6.8	154.4	10.8	153.6	11.4	195.9	3.7
Prednisone	9.2	61.7	11.5	60.7	5.0	97.5	9.8	82.3	12.0
Prednisolone	6.1	95.2	9.4	100.0	8.7	133.0	7.3	120.4	4.8
Cortisone	2.1	69.5	7.3	66.3	3.2	88.7	13.1	86.9	6.0
Boldenone	0.7	61.1	4.5	67.5	2.7	95.7	6.3	106.9	2.1
Norethisterone	2.3	42.7	2.9	44.3	3.3	73.3	9.5	76.9	2.5
Nandrolone	4.1	59.0	9.6	59.6	3.3	87.6	6.1	88.3	3.3
Progesterone	0.5	63.4	10.7	61.7	10.3	59.8	5.8	70.5	4.3

		Effluent WWTP3				Effluent WWTP4			
		100 ng L^{-1}		500 ng L^{-1}		100 ng L^{-1}		500 ng L^{-1}	
Compound	LOD[a] (ng L^{-1})	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$	Recovery (%) $n = 6$	RSD[b] (%) $n = 6$
Diethylstilbestrol	13.2	60.0	15.4	58.2	15.0	52.4	18.8	53.2	6.3
17β-estradiol	8.5	—	—	—	—	—	—	—	—
Estrone	4.1	104.2	11.4	121.2	3.7	94.7	10.2	88.9	7.1
Estriol	4.5	54.4	10.1	59.1	15.1	114.1	11.4	89.4	15.7
Norgestrel	1.6	53.9	10.2	48.5	3.8	36.1	13.8	49.1	8.1
Testosterone	1.0	30.2	8.7	47.6	3.3	46.6	12.2	60.9	7.1
Megestrol acet.	1.2	43.8	5.5	58.9	3.9	19.3	14.9	40.3	14.7
Prednisone	9.2	53.1	9.5	61.9	11.4	69.2	11.7	85.7	7.0
Prednisolone	6.1	48.9	5.2	58.7	4.6	69.2	3.5	78.0	8.1
Cortisone	2.1	34.4	4.9	42.7	5.1	48.6	7.0	65.6	3.4
Boldenone	0.7	35.5	4.9	52.5	1.9	40.9	12.8	65.6	5.8
Norethisterone	2.3	22.9	5.3	31.8	5.0	16.7	7.9	49.2	5.1
Nandrolone	4.1	25.3	8.2	41.3	3.4	24.0	4.8	59.2	5.1
Progesterone	0.5	67.7	7.2	79.6	5.9	32.2	14.1	57.0	12.7

[a] Limit of detection. [b] Relative standard deviation.

analysis was performed in gradient mode at a flow rate of 0.3 mL min^{-1}. Table 2 shows the gradient used for both the BSM and QSM.

2.4.2. Mass spectrometry conditions. The detection and identification of hormones have been developed using an ACQUITY triple quadrupole (TQD) mass spectrometer with an electrospray ionisation (ESI) interface (Waters Chromatography, Barcelona, Spain). All components were controlled using the MassLynx Mass Spectrometry Software. Electrospray ionisation parameters were as follows: the capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode, the source temperature was 150 °C, the desolvation temperature was 500 °C, and the desolvation gas flow rate was 1000 L h^{-1}. Nitrogen was used as the desolvation gas and argon was used as the collision-induced dissociation gas at a flow rate of 0.15 mL min^{-1}. The extractor and RF lens voltages were 3 V and 0.5 V, respectively, in both ionization modes.

The detailed MS/MS detection parameters for each hormone compound are presented in Table 3 and the multiple reaction monitoring (MRM) parameters were optimised by the direct injection of a 1 mg L^{-1} standard solution of each analyte into the detector at a flow rate of 10 μL min^{-1}.

3. Results and discussion

3.1. Optimization of solid-phase extraction (SPE)

3.1.1. SPE sorbent. Several authors have reported that liphophilic–hydrophilic balanced polymer and octadecyl carbon chain (C_{18}) cartridges and columns have been used to extract endocrine disrupting compounds from waste water samples.[12,20,21,29] Different sample volumes and load and wash

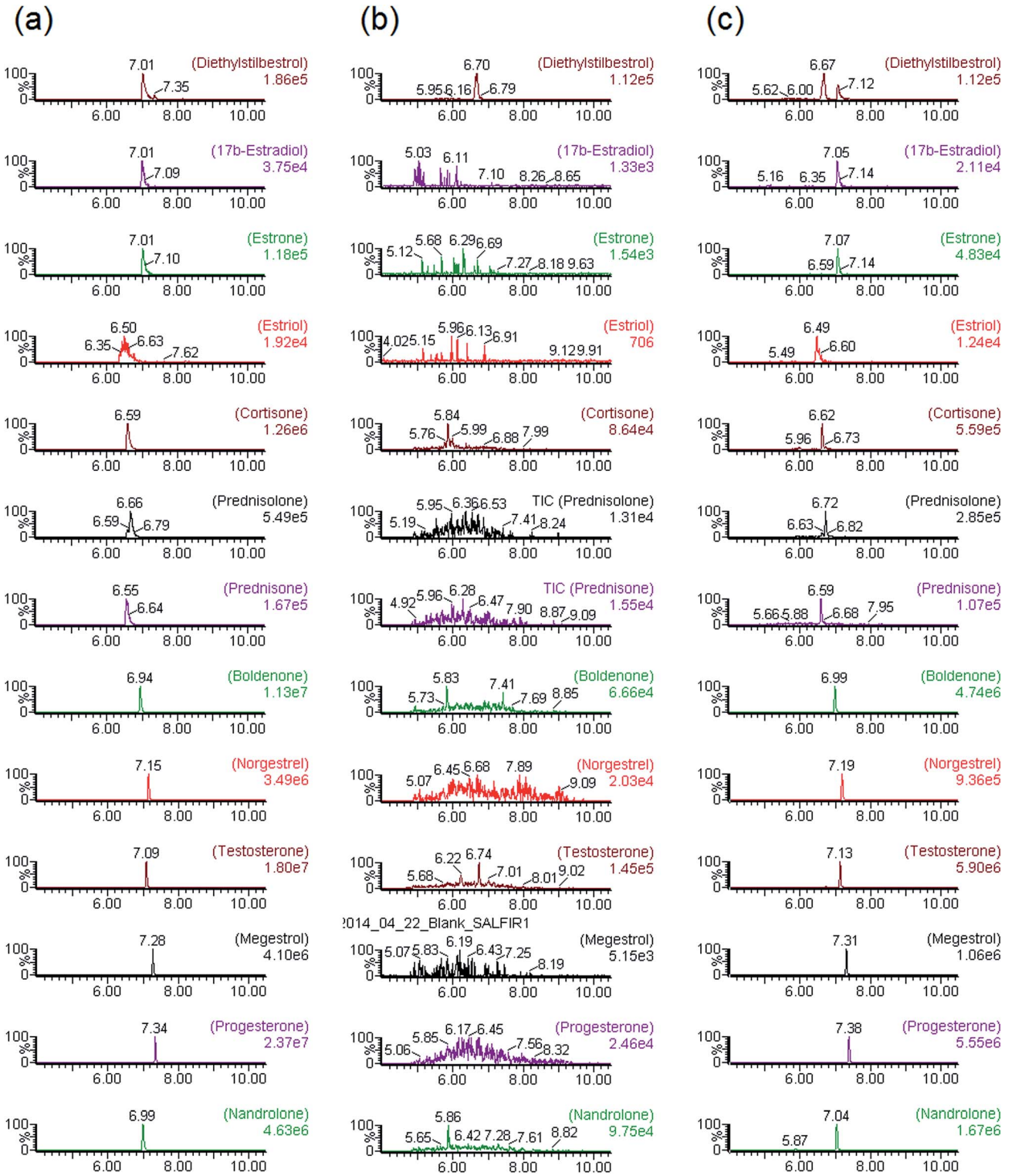

Fig. 3 Chromatograms of the target compounds in pure solvent (a), a non-spiked real sample (b) and a spiked real sample (c).

solvents were tested to achieve higher recoveries and minimize background noise generated by the sample with OASIS HLB and XBridge C_{18} SPE columns. To optimize these variables, effluent samples from the tertiary treatment of WWTP1 with a hormone concentration of 500 ng L^{-1} were used.

3.1.2. Sample volume and loading solvent. The on-line SPE system allows injection sample volumes up to 5 mL in a cycle or several cycles. In our case, we have chosen only one injection cycle because the injection time is 4 minutes as several injection cycles of sample increases the total analysis time. For this

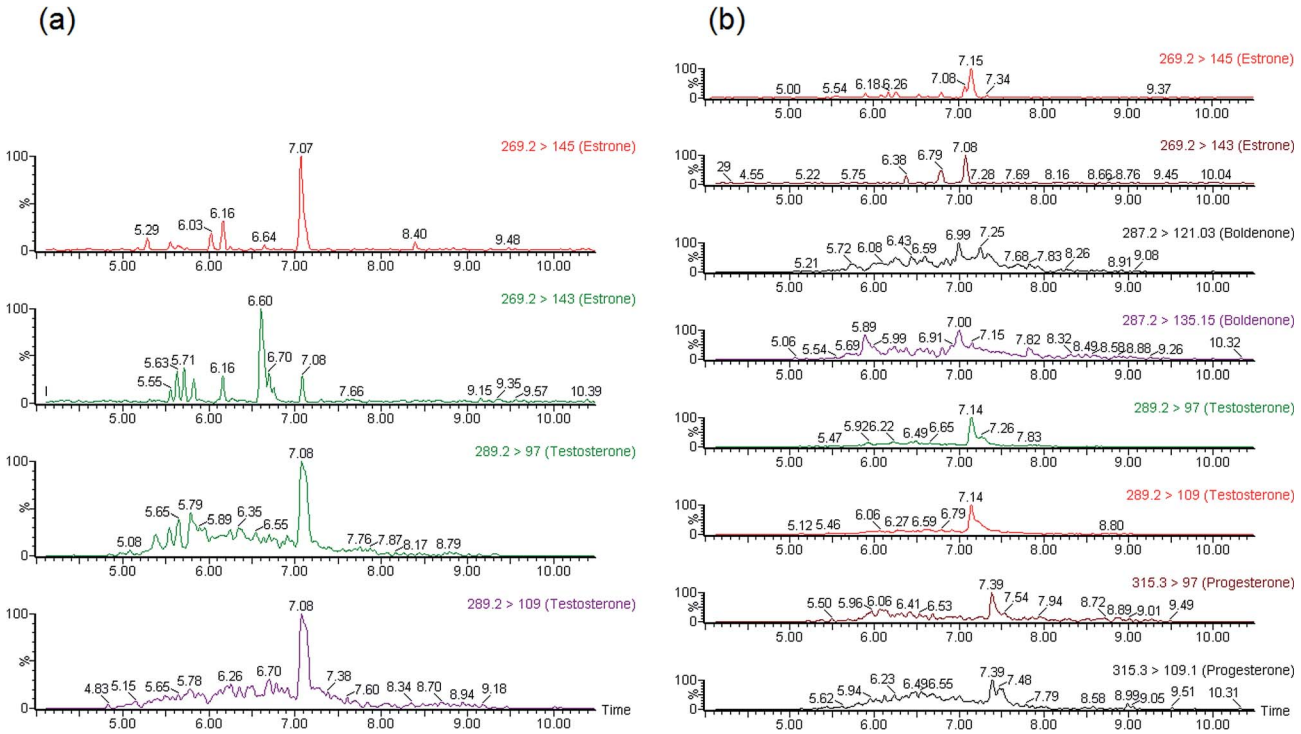

Fig. 4 Chromatograms of the compounds found in the effluent samples from WWTP3 (a) and WWTP4 (b).

reason, volumes from 1 to 5 mL have been tested to check the variations of response area of the analytes.

Another important parameter is the composition of the sample loading solvent because this solvent could improve or diminish the adsorption of analytes onto the SPE column and eliminate interferents from the matrix evaluated. The sample loading solvent is Milli-Q water and four conditions have been evaluated: with 0.1% (v/v) of NH_3 (pH = 10.1), with 0.03% (v/v) of NH_3 and 100 mM of ammonium acetate (pH = 8.1), with 0.05% (v/v) of acetic acid (pH = 3.4) and without additives (pH = 5.6).

For both SPE columns tested, maximum recoveries of most compounds were found when the sample volume is between 2 and 3 mL while volumes of 4 and 5 mL showed a significant decrease in recoveries, which may be due to the same sample producing a partial elution of the analytes. For the load phase, the pH between 3.4 and 5.6 showed better recoveries for OASIS HLB SPE columns while, XBridge C_{18} showed maximum recoveries at pH = 10.4.

3.1.3. Sample wash step. This step is very important because it allows for the elimination of interferents in the sample, thereby providing better signal to noise (S/N) ratios. With the on-line SPE system, several combinations and proportions of aqueous and organic solvents can be used in the wash step, automatically, without manipulation by the analyst. We have studied five different proportions of aqueous : organic solvents (100 : 0, 90 : 10, 80 : 20, 70 : 30 and 60 : 40) specifically methanol and water, with and without 0.1% of NH_3. Percentages greater than 40% of methanol have not been tested to avoid a co-elution of the analytes under study.

For XBridge C_{18} SPE columns, the wash step was eliminated because it caused the elution of the analytes retained in the column. For this reason, the flow rate of the wash step was reduced to the minimum that the UHPLC-MS/MS system allows for (0.01 mL min^{-1}).

For OASIS HLB SPE columns the use of a wash step without NH_3 produced higher recoveries and better S/N ratios for most compounds than the wash step with NH_3. An acid wash step has not been tested because it produced the elution of the analytes. Regarding the mixture composition of the wash step, the best results were obtained without adding methanol because the presence of an organic solvent results in deformation of the peaks for most compounds. Fig. 2 shows the peak of nandrolone as an example of this deformation and loss of the S/N ratio at different proportions of aqueous : organic solvents used in the wash step.

3.1.4. pH of the sample. The pH of the sample is an important parameter because it defines the ionised form of the analytes according to their pK_as. We have tested the acidic pH (pH = 3.5), using acetic acid, the pH of the waste water sample, that was about pH = 5.7 and the basic pH (pH = 10.4) using sodium hydroxide. The results show that the recoveries of several compounds, such as diethylstilbestrol, boldenone and nandrolone were low at pH = 3.5, whereas only two compounds (prednisone and cortisone) had a maximum recovery at this pH using the OASIS HLB SPE column. In contrast, using samples at basic or neutral pH, better recoveries were obtained for most compounds with both types of SPE columns. There were no significant differences between pH = 5.7 and pH = 10.4, but at basic sample pH the recoveries were slightly higher for ten of

Table 5 Concentrations at ng L^{-1} of compounds found in effluent samples[a]

Compound	WWTP2	WWTP3	WWTP4
Estrone	nd[b]	<LOQ	14.0 ± 4.9
Testosterone	nd[b]	52.8 ± 1.2	12.6 ± 3.8
Boldenone	nd[b]	nd[b]	5.6 ± 0.3
Progesterone	nd[b]	nd[b]	3.1 ± 0.4

[a] $n = 3$. [b] Not detected.

the fourteen compounds under study. Using the sample at pH = 10.4, the recoveries were, in all cases, over 63.5% for OASIS HLB SPE columns.

3.1.5. Desorption step. Desorption of the analytes from the SPE column was performed using water with 0.1% of NH_3 and methanol, which is the same mobile phase used for chromatographic separation. This desorption step was performed in gradient mode as with the chromatographic separation in the analytical column. Notably, if the analytes can be eluted from the UHLPC column using the solvents in gradient mode, they will be eluted completely from the SPE column, because the retention capabilities are either similar or lower than the retention capabilities of the analytical columns. The use of a gradient mode to elute the analytes is not possible with conventional off-line extraction using SPE cartridges in a manifold. For these reasons, the desorption step in on-line SPE uses smaller volumes of organic solvent and shorter times of extraction, achieving better signal to noise ratios and therefore, lower limits of detection.

Once all the parameters were optimised, we selected the OASIS HLB SPE column, because with this SPE column, the recoveries were higher (over 60% for most compounds) than the recoveries obtained with XBridge C_{18} columns (between 9 and 57%).

3.2. Analytical parameters and quality control

Linearity, recovery, repeatability, limits of detection and limits of quantification were evaluated for each waste water sample (Table 4) using OASIS HLB SPE columns under the optimum extraction conditions. External calibration curves were prepared from 0.5 to 400 μg L^{-1} of each compound. Moreover, three surrogate standards (estrone D2, testosterone D3 and progesterone D9), at a fixed concentration of 200 μg L^{-1}, were added to each calibration level. The linearity was calculated using the relationship between areas and concentrations of compounds and surrogates with excellent correlation coefficients (r^2) higher than 0.990.

The repeatability and recoveries were studied intra-day using six samples of contaminated waste water with hormones at low and high concentration levels (100 and 500 ng L^{-1}). These analytical parameters have been studied in samples from the effluent of the tertiary treatment of WWTP1 and in samples from WWTP2, WWTP3 and WWTP4 effluents.

The recoveries calculated are a combination of extraction recoveries and matrix effects on the analytes in the detector due to the impossibility of separating the extraction and identification processes. For most compounds, the recoveries ranged from 50 to 90%, except prednisolone and megestrol acetate that showed recoveries between 120 and 150%, produced by an enhancement of signal from matrix effects. Only diethylstilbestrol and norgestrel presented recoveries below 40%. The waters of WWTP3 and WWTP4 come from a big population and undergo a traditional water treatment, and the recoveries of this waste water were worse than the recoveries of the samples of the other two WWTPs, which work with a membrane bioreactor technology and have a tertiary process to purify the waste water.

The relative standard deviations were satisfactory and similar for most compounds in all samples. At a concentration of 100 ng L^{-1} the RSD was slightly to moderately higher than that at a concentration of 500 ng L^{-1}. In all cases, the RSDs were lower than 18%.

The limit of detection (LOD) and the limit of quantification (LOQ) for each compound were calculated from the signal to noise ratio of each individual peak. The LOD was defined as the lowest concentration that gave a signal to noise ratio that was greater than 3. The LOQ was defined as the lowest concentration that gave a signal to noise ratio that was greater than 10. The LODs and LOQs ranged from 0.5 to 13.2 ng L^{-1} and from 1.66 to 44 ng L^{-1}, respectively. These limits are similar to other studies that used off-line SPE with large sample volumes.[19,30,31]

Finally, the method shows a good selectivity as can be seen in Fig. 3. This figure shows the chromatograms of a standard, a non-spiked and a spiked sample.

3.3. Analysis of selected compounds in waste water samples

The on-line SPE-UHPLC-MS/MS developed method was applied for the detection of target analytes in different waste water

Table 6 Parameters of other on-line SPE methods used for the determination of steroid hormones in waste water samples

Compounds studied	Sample volume	Analysis time	Average recoveries (%)	Reference
Estrogens	1 mL	13 min	79–95	Salvador *et al.*[34]
Estrogens progestogens	1 mL	15 min	85–110	Viglino *et al.*[32]
Estrogens	50 mL	45 min	86–107	Wang *et al.*[33]
Estrogens, progestogens	10 mL	15 min	71–95	Fayad *et al.*[28]
Estrogens androgens	50 mL	40 min	31–120	Guo *et al.*[18]
Estrogens	2.5 mL	10 min	80–98	Ciofi *et al.*[20]
Estrogens, androgens, progestogens, glucocorticoids	2 mL	15 min	43–95	This study

samples from WWTPs on the island of Gran Canaria (Spain) to check the efficiency of this method. The samples were collected from the effluent of the tertiary treatment of one WWTP and from effluent of three waste water treatment plants that use a membrane bioreactor and activated sludge as treatments. The samples were collected in May of 2014 and January of 2015. To evaluate the matrix effect, three surrogate standards (estrone D2, testosterone D3 and progesterone D9) were added before the extraction process. They could not be added after the on-line extraction due to the configuration of the UHPLC-MS/MS system. To quantify the concentrations of the compounds, the ratios between the peak area of the quantification ions and the peak area of the surrogate standards were used. Fig. 4a and b show the chromatograms of the compounds found in the effluent samples from WWTP3 and WWTP4.

In the effluent of WWTP3, only estrone at a concentration below the quantification limit and testosterone at a concentration of about 50 ng L^{-1} were detected. In the effluent sample of WWTP4 four steroid hormones were detected. Progesterone and boldenone were detected at concentrations below 5.6 ng L^{-1}, while estrone and testosterone concentrations ranged from 12.6 to 14 ng L^{-1}. The concentrations of each compound found are shown in Table 5.

In the effluent samples from WWTP2, any compound under study that was not detected can be interpreted as a removal of the hormone by the treatment used at WWTP. Several authors have stated this removal in different waste water treatment plants all over the world.[13,15,21,32]

Table 6 summarizes the studied compounds, sample volume, analysis time and recoveries obtained in other on-line SPE methods used for the determination of steroid hormones in waste water samples. The whole analysis (extraction and determination) usually takes between 10 and 20 minutes as in the method developed in this paper. However, the studies of some authors, Guo *et al.*[18] and Wang *et al.*,[33] present analysis time up to 45 minutes. Another important parameter is the sample volume. In this article, 2 mL of waste water are analyzed, which is a similar volume to that used in other studies by Viglino *et al.*,[32] Ciofi *et al.*[20] and Salvador *et al.*[34] These volumes minimize the analysis time as can be seen in Table 6. The recoveries obtained in this article are in the range of the recoveries obtained by other authors. Nevertheless, the main drawback of other on-line SPE methods is the type of steroid hormone that they determine. Ciofi *et al.*, Wang *et al.* and Salvador *et al.*[20,33,34] developed on-line SPE methods only for estrogens, while Guo *et al.*, Fayad *et al.* and Viglino *et al.*[18,28,32] have optimized their methods for estrogens and progestogens or androgens. The on-line SPE method developed in this paper is suitable for estrogens, androgens, progestogens and glucocorticoids. In addition, for the glucocorticoids, this is the first on-line SPE method for their determination, because they have been usually extracted from environmental samples using offline procedures.

4. Conclusions

A selective, sensitive and appropriate on-line SPE-UHLPC-MS/MS method for the determination of hormones in waste water samples at low ng L^{-1} concentrations was developed. All of the parameters involved in the extraction step, such as the sorbent type, sample volume, loading solvent, wash solvent and pH of the sample, have been optimized to achieve maximum recoveries. The developed method performs a whole process of extraction, identification and determination of four types of steroid hormones in 15 minutes, is fully automated, and requires only 2 mL of water sample; the parallel work with the samples increases efficiency.

The developed method offers low limits of detection and quantification (LODs and LOQs ranging from 0.5 to 13.2 ng L^{-1} and from 1.7 to 44 ng L^{-1}, respectively) and high selectivity, which are important in the analysis of these emerging pollutants in environmental and complex matrices. The recoveries have been satisfactory, ranging between 50 and 90% for most compounds in effluent samples, and all of them with RSDs lower than 15% in most cases.

The application of this method to real samples has been satisfactory and four hormones (one oestrogen, two androgens and one progestogen) have been determined in effluent samples with concentrations ranging from 3 to 52 ng L^{-1}. No hormones were detected in the effluent sample of the waste water treatment plant that uses membrane bioreactor technology.

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References

1 J. R. Colman, D. Baldwin, L. L. Johnson and N. L. Scholz, *Aquat. Toxicol.*, 2009, **91**, 346–354.
2 P.-D. Hansen, H. Dizer, B. Hock, A. Marx, J. Sherry, M. McMaster and C. Blaise, *TrAC, Trends Anal. Chem.*, 1998, **17**, 448–451.
3 Ø. Øverli, S. Kotzian and S. Winberg, *Horm. Behav.*, 2002, **42**, 53–61.
4 J. D. DiBattista, H. Anisman, M. Whitehead and K. M. Gilmour, *J. Exp. Biol.*, 2005, **208**, 2707–2718.
5 R. D. Blackburn, A. Cunkelman and V. M. Zlidar, *Popul. Rep. A*, 2000, **28**, 1–16, 25–32.
6 M. Y. Abdel-Rahman and W. W. Hurd, *MedScape*, 2014, www.medscape.com.
7 European Commission, Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC, 1996.
8 Y. Xu, N. Xu, N. R. Llewellyn and H. Tao, *Environ. Sci.: Processes Impacts*, 2014, **16**, 262–270.
9 Z. Liu, Y. Kanjo and S. Mizutani, *Sci. Total Environ.*, 2009, **407**, 4975–4985.
10 H. Tomšíková, J. Aufartová, P. Solich, L. Nováková, Z. Sosa-Ferrera and J. J. Santana-Rodríguez, *TrAC, Trends Anal. Chem.*, 2012, **34**, 35–58.

11 I. Robinson, G. Junqua, R. V. Coillie and O. Thomas, *Anal. Bioanal. Chem.*, 2007, **387**, 1143–1151.
12 H. Chang, Y. Wan, S. Wu, Z. Fan and J. Hu, *Water Res.*, 2011, **45**, 732–740.
13 G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Mancini, R. Mastropasqua, M. Nazzari and R. Samperi, *Sci. Total Environ.*, 2003, **302**, 199–209.
14 T. Vega-Morales, Z. Sosa-Ferrera and J. J. Santana-Rodríguez, *J. Hazard. Mater.*, 2010, **183**, 701–711.
15 H. Chang, J. Hu and B. Shao, *Environ. Sci. Technol.*, 2007, **41**, 3462–3468.
16 R. Guedes-Alonso, C. Afonso-Olivares, S. Montesdeoca-Esponda, Z. Sosa-Ferrera and J. J. Santana-Rodriguez, *SpringerPlus*, 2013, **2**, 1–8.
17 N. H. Tran, J. Hu and S. L. Ong, *Talanta*, 2013, **113**, 82–92.
18 F. Guo, Q. Liu, G. Qu, S. Song, J. Sun, J. Shi and G. Jiang, *J. Chromatogr. A*, 2013, **1281**, 9–18.
19 R. Guedes-Alonso, Z. Sosa-Ferrera and J. J. Santana-Rodriguez, *J. Anal. Methods Chem.*, 2013, **2013**, e210653.
20 L. Ciofi, D. Fibbi, U. Chiuminatto, E. Coppini, L. Checchini and M. Del Bubba, *J. Chromatogr. A*, 2013, **1283**, 53–61.
21 S. Liu, G.-G. Ying, J.-L. Zhao, F. Chen, B. Yang, L.-J. Zhou and H. Lai, *J. Chromatogr. A*, 2011, **1218**, 1367–1378.
22 S. Rodriguez-Mozaz, M. J. López de Alda and D. Barceló, *J. Chromatogr. A*, 2004, **1045**, 85–92.
23 M. Pedrouzo, F. Borrull, E. Pocurull and R. M. Marcé, *Talanta*, 2009, **78**, 1327–1331.
24 T. Vega-Morales, Z. Sosa-Ferrera and J. J. Santana-Rodríguez, *J. Chromatogr. A*, 2012, **1230**, 66–76.
25 B. Lei, S. Huang, Y. Zhou, D. Wang and Z. Wang, *Chemosphere*, 2009, **76**, 36–42.
26 A. Arditsoglou and D. Voutsa, *Mar. Pollut. Bull.*, 2012, **64**, 2443–2452.
27 N. Andrási, B. Molnár, B. Dobos, A. Vasanits-Zsigrai, G. Záray and I. Molnár-Perl, *Talanta*, 2013, **115**, 367–373.
28 P. B. Fayad, M. Prévost and S. Sauvé, *Talanta*, 2013, **115**, 349–360.
29 H. Chang, S. Wu, J. Hu, M. Asami and S. Kunikane, *J. Chromatogr. A*, 2008, **1195**, 44–51.
30 M. Kuster, D. A. Azevedo, M. J. López de Alda, F. R. Aquino Neto and D. Barceló, *Environ. Int.*, 2009, **35**, 997–1003.
31 T. Anumol, S. Merel, B. O. Clarke and S. A. Snyder, *Chem. Cent. J.*, 2013, **7**, 104.
32 L. Viglino, K. Aboulfadl, M. Prévost and S. Sauvé, *Talanta*, 2008, **76**, 1088–1096.
33 S. Wang, W. Huang, G. Fang, J. He and Y. Zhang, *Anal. Chim. Acta*, 2008, **606**, 194–201.
34 A. Salvador, C. Moretton, A. Piram and R. Faure, *J. Chromatogr. A*, 2007, **1145**, 102–109.
35 *Advanced Chemistry Development (ACD/Labs) Software V11.02*, Scifinder, Chemical Abstracts Service, Columbus, Ohio, USA, 2013.