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Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography–diode array detection–mass spectrometry

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Abstract

In this study, a procedure for the determination of various naturally occurring hormones and of some related synthetic chemicals, commonly used for birth control and treatment of certain hormonal disorders and cancers, in water is described. The procedure includes solid-phase extraction of 0.5 l of water and subsequent analysis of the extract by liquid chromatography with diode array detection and mass spectrometric detection in series (LC–DAD–MS). DAD has been performed at 197, 225, and 242 nm for quantification and confirmatory identification purposes. For MS detection two interfaces — electrospray and atmospheric pressure chemical ionization — in both the positive and the negative ion mode have been tested and the MS parameters influencing the MS signal optimized. DAD and MS have been intercompared for selectivity, sensitivity, precision, and linearity of response. Selected conditions have been applied to the determination of six estrogens (17β-estradiol, estriol, estrone, ethynylestradiol, mestranol, and diethylstilbestrol) and four progestogens (progesterone, levonorgestrel, norethindrone and ethynodiol diacetate) in several types of water bodies, including sewage influents and effluents, surface water and drinking water. Recoveries greater than 83% and detection limits in the ng/l range have been achieved for most compounds.

Keywords: Detection, LC; Water analysis; Environmental analysis; Hormones; Estrogens; Progestogens

1. Introduction

In the past few years a number of papers have highlighted the potentially dangerous consequences to human and wildlife of the presence of endocrine disrupting compounds (EDCs) in the aquatic environment [1–7]. An endocrine disrupter is defined as ''an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function'' [8]. EDCs constitute, therefore, a class of substances which is not defined by chemical nature but by biological effect [9]. Thus, a wide variety of pollutants which have been reported to disrupt normal pathways in animals, including pesticides [10], polycyclic aromatic hydrocarbons [11], phthalate plasticizers [12], certain polychlorinated biphenyls, dioxins, furans, alkylphenols, synthetic steroids, and natural products such as phytoestrogens, are collectively referred to as EDCs [1,13,14].

Of particular concern is the group of synthetic steroids. This concern raises in part from the increasing use of birth-control pills, formulated with exogeneous estrogenic and progestational chemicals that show high physiological activity at very low concentrations and have been associated to certain

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alarming effects on reproduction and developmental processes, such as feminization, decreased fertility, or hermaphroditism [1–3,15–19].

The estrogen content in such preparations, used in the management of menstrual and menoupausal disorders as well as for contraception, is usually in the range 20 to 50 μg daily [20]. As for the progestogenic content, it varies depending on the type of contraceptive. Thus, in combined oral formulations the progestogenic content is in the range 0.25 to 2 mg daily whereas in progestogen-only contraceptives it is lower (30–500 μg daily).

Other than contraception, the uses of estrogens can largely be put into three main groups: The management of the menopausal and postmenopausal syndrome (its widest use); physiological replacement therapy in deficiency states; and the treatment of prostatic cancer and of breast cancer in postmenopausal women.

Likewise, progestogens are used in the treatment of several other conditions such as infertility, endometriosis, in the management of certain breast and endometrial cancers, and either alone or in combination with estrogens in the treatment of menstrual disorders, among others.

Progestogens are commonly added to estrogens to protect against endometrial hyperplasia and cancer because unopposed estrogen therapy may cause endometrial proliferation.

The therapeutic doses required in the treatment of many of these diseases are often much larger than those employed in contraception. Thus, norethindrone is administered at doses as high as 60 mg daily in metastatic breast cancer, and intravaginal or intrarectal doses of progesterone of 200 to 400 mg twice daily are used in the treatment of puerperal depression.

Synthetic estrogens and progestogens show great differences in relation to excretion rates, water solubility and biological catabolism [6,21]. In general, they are readily adsorbed from the gastro-intestinal tract and through the skin or mucous membranes, and metabolized in the liver with some undergoing enterohepatic recycling. Excretion of unchanged drug and the less active metabolites is in the urine and a small amount in the feces [20], in urine usually as water-soluble conjugates and in feces as ''free'' estrogens [22]. Human excretion of estrogens, which has been estimated to be around 2.7 mg/l on a daily basis, is thought to be the principal source of these type of compounds in the aquatic environment.

There is little information in the literature on the fate and persistence of synthetic ovulation-inhibiting hormones in the aquatic environment. Natural and synthetic estrogens and progestogens entering wastewater treatment plants from urban and industrial discharges are subject to a variety of treatment processes of varying efficiency and in some cases they are finally released into surface waters [6,23–27].

Moreover, it is been reported that the less active conjugated forms can be deconjugated during wastewater treatment and in the environment to generate the more potent parent compound [28–30].

On the other hand, and as a consequence of the use of sewage sludges in agriculture, compounds that were effectively removed in the water treatment process may also reach surface waters through runoff [31,32].

By the mentioned ways, these potentially dangerous substances can reach the aquatic environment, cause adverse physiological effects to wildlife, and enter the alimentary chain and reach humans [4].

The presence of both natural and synthetic estrogens and progestogens in the various types of water has, in most instances, been reported to occur at the low ng per liter range up to the tens of nanograms per liter range [4,5,22,28,30,33–35] and only in a few occasions concentrations have been higher reaching μg/l levels [23,27,36]. In view of these levels and taking into account the doses employed in pharmaceutical preparations as well as the human excretion concentrations seems logical to think that the human environmental exposure to these compounds is negligible. However, neither the concentrations in water at which these compounds can cause adverse biological responses in wild life or humans nor the synergism that may occur because of the presence of many other pollutants with estrogenic activity in the aquatic medium are well-known yet [6,33,37].

Thus, monitoring of these compounds in water is of great importance, and in fact, in the last few years an increasing number of studies have been conducted to developed analytical procedures to estimate to what extent these compounds are present in the aquatic environment.

Most of these studies have been limited to the

determination of just a few estrogens or progestogens and the analytical procedures have included in most cases either biological techniques [5,22,23,34,35, 38,39] or gas chromatography–mass spectrometry (GC–MS) after a more or less complicated method of extraction with a solid-phase extraction (SPE) in octadecylsilane (ODS) supports as a usual first step [4,28,30,33,36,40].

On the contrary, liquid chromatography (LC) has only been employed in a few occasions [22,38,41,42] regardless of its advantages with respect to the already mentioned techniques. Thus, unlike GC–MS, LC enables the determination of both steroids and conjugates, without derivatization, and is not limited by such factors as nonvolatility and high molecular weight. Likewise, LC–MS, though not as sensitive as some biological techniques such as immunoassays, offers the advantage of being more specific, allowing the simultaneous screening of a wide range of micropollutants, and not being limited by the availability of specific antisera.

In this study, an analytical procedure for the determination of several natural and synthetic estrogens and progestogens in water is presented. The procedure includes SPE extraction of the water sample and subsequent analysis of the extract by LC–diode array detection (DAD)–MS. To demonstrate the applicability of the method several types of water were analyzed by using this method. Target analytes (see Fig. 1) were selected based on their abundance in the human body in the case of the natural hormones, and based on the extent of their use in pharmaceutical formulations in the case of the synthetic compounds.

Thus, the group of target natural hormones included estradiol, the most potent mammalian estrogenic hormone, estrone and estriol, the main metabolites of estradiol which possess considerably less biological activity, and progesterone.

The group of synthetic chemicals was represented by levonorgestrel, nortehindrone and ethynodiol diacetate, the progestogens most commonly used in progestogen-only oral contraceptives, ethynyl estradiol and mestranol, the estrogens most commonly used in combined oral contraceptives, and diethylstilbestrol.

Diethylstilbestrol has been extensively used in the past in estrogenic hormone therapy in the prevention of miscarriage in humans (to prevent spontaneous abortion) and as a growth promotant in livestock [20]. However, its use for growth-promoting purposes is controversial and in some countries has been banned. The US Food and Drug Administration (FDA), for instance, banned its use in 1979.

Conjugated forms of the steroids, such as glucuronides or sulphates, were not analyzed because of their lessened biological activity.

Even though the US Environmental Protection Agency has mentioned that the effects on reproduction and development of hormone-modulating pollutants should be of greater concern than their ability to cause cancer [2], it may be worth saying that diethylstilbestrol is listed as a known carcinogen, and progesterone and all the estrogens analyzed with the exception of estriol have been cataloged as substances that may reasonably be anticipated to be carcinogens [20].

2. Experimental

2.1. Reagents and materials

Pure standards of both natural and synthetic estrogens and progestogens, including the internal standard, [$^2H_2$]17β-estradiol, were purchased as powders from Sigma (St. Louis, MO, USA). Stock standard solutions for each of the analytes were prepared at 10 g/l in methanol. Working solutions of the individual standards and of mixtures of all of them were prepared at various concentrations by appropriate dilution of the stock solutions in methanol.

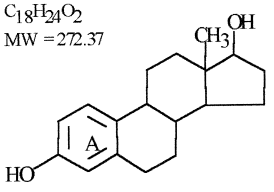
HPLC-grade solvents acetonitrile, methanol, and water, and sulfuric acid proanalysis grade were purchased from Merck (Darmstadt, Germany).

2.2. Sampling

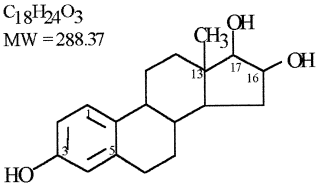
Samples were collected in Pyrex borosilicate amber glass containers. Each bottle was rinsed with tap water and with high-purity water prior to sample addition. Sample preservation was accomplished by storing the bottles at 4°C immediately after sampling. Extraction is carried out as soon as possible in order to avoid addition of chemical preservatives. However, if extraction does not take place within 48 h

ESTROGENS

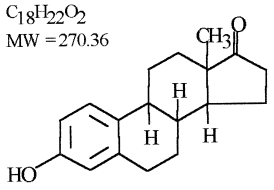
Estradiol
$C_{18}H_{24}O_2$
MW = 272.37



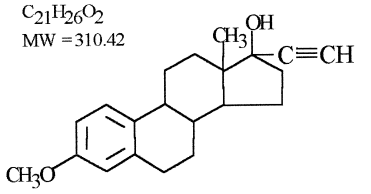
Estriol
$C_{18}H_{24}O_3$
MW = 288.37



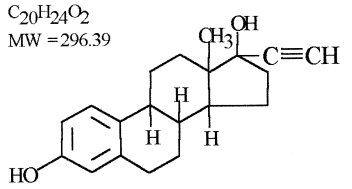
Estrone
$C_{18}H_{22}O_2$
MW = 270.36



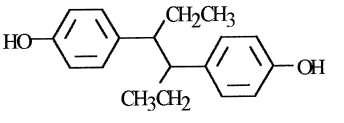
Mestranol
$C_{21}H_{26}O_2$
MW = 310.42



Ethinyl Estradiol
$C_{20}H_{24}O_2$
MW = 296.39

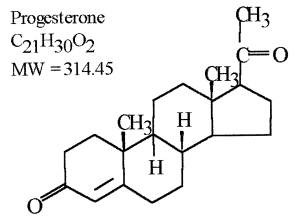


Diethylstilbestrol
$C_{18}H_{20}O_2$
MW = 268.34

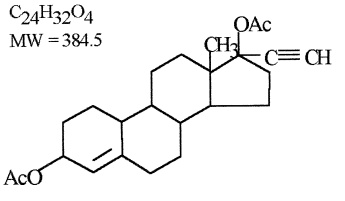


PROGESTOGENS

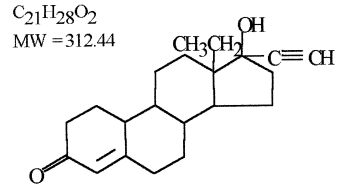
Progesterone
$C_{21}H_{30}O_2$
MW = 314.45



Ethynodiol Diacetate
$C_{24}H_{32}O_4$
MW = 384.5



Levonorgestrel
$C_{21}H_{28}O_2$
MW = 312.44



Norethindrone
$C_{20}H_{26}O_2$
MW = 298.41

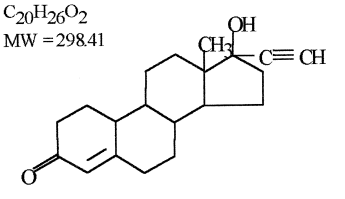


Fig. 1. Molecular structure of the natural and synthetic estrogens and progestogens analyzed.

after collection sulfuric acid is added until pH 3 to prevent biological degradation.

Several types of water, including a sewage treatment plant (STP) influent and effluent, river water, and drinking water, collected in the area of Catalonia (NE Spain), were analyzed in order to demonstrate the applicability of the method.

Both the influent and the effluent water were collected as 24 h composite samples from a STP located in Piera (Barcelona, Spain), receiving mostly urban waste waters, in June 1999. The STP works consist of a primary settlement followed by a biological treatment. It receives an average of 4000 m^3/day (maximum flow 848 m^3/h), from several cities counting with a total population of 13 000–18 000 inhabitants. This plant is routinely controlled by the Water Authorities who determine an average biological oxygen demand (BOD) and suspended solids removal of 93–94%.

The river water was collected in May 1999 in the Anoia river, a highly polluted river that receives effluents from various STPs including the previously described plant, at approximately 20 km downstream from the discharge point of the STP located in Piera.

The drinking water sample was collected in July 1999 at the outlet of the water treatment plant that supplies water for human consumption to most parts of Barcelona city.

Grab sampling was used for collection of the river water and the drinking water.

2.3. Sample preparation procedure

The overall schematic procedure is presented in Fig. 2.

Prior to extraction, samples were filtered through glass fiber filters (0.45 μm pore size).

Extraction was performed using an automated sample processor ASPEC XL (Automated Sample Preparation with Extraction Columns) fitted with a 817 switching valve and an external 306 LC pump, for selection and dispensing of samples, respectively, through the SPE cartridges, from Gilson (Villiers-le-Bel, France).

Some preliminary experiments were run in order to test critical factors affecting the extraction efficiency of the procedure, including selection of the cartridge, sample volume, the solvent and volume used for elution, and pH adjustment of the water sample.

For cartridge and sample volume selection, varying sample volumes (250, 500, and 1000 ml) of distilled water spiked with each of the analytes at 10 μg/l were percolated at 5 ml/min through a variety of disposable SPE cartridges—LiChrolut EN (200 mg) and LiChrolut RP-18 (500 mg), both from Merck, and Isolut ENV from International Sorbent Technology (Cambridge, UK)—. In all instances conditioning of the cartridges was accomplished by passing 7 ml of acetonitrile, 5 ml of methanol, and 5 ml of LC-grade water at a flow-rate of 3 ml/min.

After sample loading, cartridges were dried with a Baker LSE 12G apparatus (J.T. Baker, Deventer, Netherlands) connected to a vacuum system at −15 p.s.i. (1 p.s.i.=6894.76 Pa). The drying step took 20–30 min.

Elution was performed by passing a total volume of 10 ml of acetonitrile, which was dispensed in two steps (2×5 ml) with a 5 min delay between them. The extracts obtained were then blown down to dryness under nitrogen and reconstituted with methanol to a final volume of 0.5 ml for subsequent LC–DAD–MS.

For selection of the solvent and volume used for elution, an appropriate number of preconditioned LiChrolut RP-18 (500 mg) cartridges (sorbent selected as a result of the preceding study) was directly loaded with 0.5 ml of a standard solution containing the mixture of all the analytes at 10 μg/ml, i.e., the equivalent to loading the cartridges with 500 ml of the above spiked water. Various elution volumes (2×3, 2×4, and 2×5 ml) of dichloromethane, acetonitrile and methanol were then passed through the various cartridges and the corresponding extracts evaporated and reconstituted as described above for subsequent LC–DAD–MS analysis. Acetonitrile, dispensed in two steps of 4 ml each, gave comparatively better results (results not shown).

The pH effect on the extraction efficiency was also evaluated by adjusting the water sample pH to 7 and 3 with sulfuric acid prior to extraction, but no differences were encountered in the recoveries obtained with both approaches.

The optimized extraction–preconcentration procedure, which set a sample volume of 500 ml, had a concentration factor of 10^3.

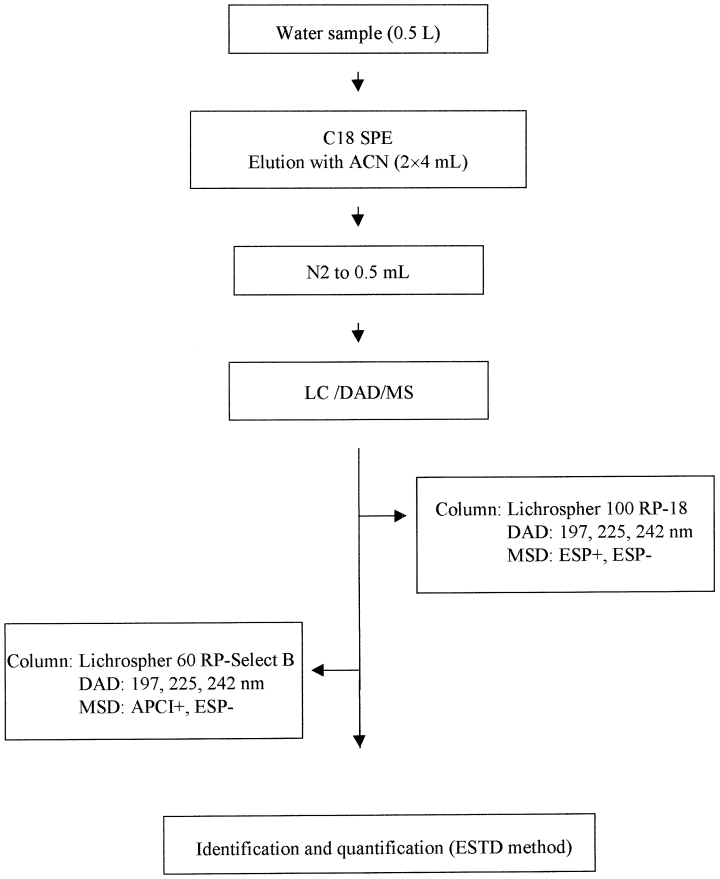


Fig. 2. Scheme of the analytical procedure. ACN = Acetonitrile; MSD = mass-selective detection.

2.4. LC–DAD–MS analysis

The HPLC system consisted of an HP 1100 autosampler with the volume injection set to 20 μl and an HP 1090 A LC pump both from Hewlett-Packard (Palo Alto, CA, USA). Separation is achieved, in first instance, on a LiChrospher 100 RP-18 column (250×4 mm, 5 μm) preceded by a guard column (4×4 mm, 5 μm) of the same packing material from Merck (Darmstadt, Germany). Samples suspected to contain target analytes are secondarily analyzed in another column with different selectivity towards the analytes: A LiChrospher 60 RP-Select B (250×4 mm, 5 μm) preceded by a guard column (4×4 mm, 5 μm) of the same packing material from Merck. Table 1 shows the LC gradient elution conditions used with each column. All chromatographic solvents were degassed with helium.

Detection was performed with a diode array detector model 1040M coupled in series with a mass spectrometer HP 1100 MSD API-ES, all from Hewlett-Packard.

Table 1
LC gradient elution conditions for separation of selected estrogens and progestogens in LiChrospher 100 RP-18 and LiChrospher 60 RP-Select B. Flow: 1 ml/min. Mobile phase components: A = acetonitrile; B = water

LiChrospher 100 RP-18			LiChrospher 60 RP-Select B		
0 min	10% A	90% B	0 min	30% A	70% B
40 min	100% A	0% B	5 min	30% A	70% B
42 min	100% A	0% B	30 min	100% A	0% B

Table 2
Optimized mass spectrometric detection conditions[a]

	ESP−	ESP+	APCI−	APCI+
Nebulizer pressure (p.s.i.)	55	55	60	60
Drying gas temperature (°C)	300	350	350	300
Drying gas flow (l/min)	13	13	4	9
Capillary voltage (V)	3500	6000	5000	3000
Fragmentor (V)	110	90	130	80
Vaporizer temperature (°C)			350	300
Corona (μA)			25	4

[a] Nebulizing and drying gas: Nitrogen.

UV chromatograms were recorded at 197, 225, and 242 nm. UV spectra from 190 to 600 nm were also recorded for peak purity assessment and to aid their identification through the comparison with libraries created for that purpose.

MS detection was performed by using two different interfaces: Electrospray (ESP) and atmospheric pressure chemical ionization (APCI). Both ESP and APCI, in the positive ion mode of operation, were used for the group of progestogens, ESP as a first option to be used with the LiChrospher 100 RP-18 column, and APCI as a second option to be used with the LiChrospher 60 RP-Select B column for confirmation of previously determined positive samples. ESP in the negative ion mode was used for the group of estrogens in both instances. Table 2 lists the optimized operational conditions used in each case. Chromatograms were recorded under the time-scheduled selected ion monitoring (SIM) conditions shown in Table 3. Nitrogen was used as nebulizing and drying gas.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

As mentioned before, prior to extraction, all samples were filtered through glass fiber filters (0.45 μm pore size). Filtration could be side-stepped in the case of clean waters such as drinking water or ground-water. But in the case of waters with high levels of suspended solids or turbidity, such as

Table 3
Time-scheduled SIM conditions and base peak from the LC–MS analysis of selected estrogens and progestogens in water samples using two different LC columns

Compound	LC column		MS ion mode	M_r	ESP		APCI	
	RP-18 time (min)	RP-SelectB time (min)			m/z	Base peak	m/z	Base peak
Estriol	0.00	0.00	NI	288	287	[M–H]−	287	[M–H]−
Estradiol	18.00	13.00	NI	272	271	[M–H]−	271	[M–H]−
Ethinyl estradiol	–	–	NI	296	295	[M–H]−	295	[M–H]−
Estrone	–	–	NI	270	269	[M–H]−	269	[M–H]−
Diethylstilbestrol	–	18.00	NI	268	267	[M–H]−	267	[M–H]−
Mestranol	–	–	–	310	ND	ND	ND	ND
Norethindrone	0.00	0.00	PI	298	321	[M+Na]+	299	[M+H]−
Levonogestrel	24.30	13.00	PI	312	335	[M+Na]+	313	[M+H]−
Progesterone	29.40	20.50	PI	314	337	[M+Na]+	315	[M+H]−
Ethynodiol diacetate	34.00	22.00	PI	384	347	[M–2CH_3CO+2Na+3H]+	265	[M–2AcO+H]+

surface water and waste-water, filtration turned out to be absolutely necessary in order to avoid subsequent clogging of the solid support used for extraction.

The selection of the sample volume, finally determined to be 500 ml, responded to time-saving considerations, and not to breakthrough values. Table 4 lists the recovery percentages obtained from the analysis of various sample volumes of distilled water, spiked at 10 μg/l with each analyte, extracted with three different SPE cartridges. As it can be seen, all three sorbents exhibited a general similar retention behavior towards all analytes but estriol. Thus, with the exception of ethynodiol diacetate, which was not recovered at all from any of the sorbents tested, and estriol, which showed different affinity for the various solid-phases, all the remaining compounds were trapped and desorbed from the three sorbents to a similar extent and with recoveries always above 50%. In the case of the estriol, by the contrary, satisfactory recoveries were only achieved with LiChrolut RP-18 cartridges, and the sample volume did not have any effect. In the other sorbents tested, i.e., LiChrolut EN and Isolut ENV, the estriol experimented, regardless of the sample volume, breakthrough as a consequence of its capacity factor. Consequently, LiChrolut RP-18 and 1000 ml could have been selected as solid support and sample volume, respectively. However, the sample volume was finally determined to be 500 ml in order to diminish the time invested during the extraction step and overall because the filtration step is often very time-consuming.

Other factors involved in the extraction–preconcentration procedure, such as the loading flow-rate of the sample, which could lead to lower recoveries of the compounds with retention volumes close to the sample volume due to non-equilibrium processes, or the drying step, which could yield lower recoveries of the more volatile compounds, were not further checked, because of the already satisfactory recoveries obtained for most analytes under the initial conditions tested.

3.1.2. LC–DAD–MS

Chromatographic separation was achieved with two different columns. The LiChrospher 100 RP-18 column was preferred over the LiChrospher 60 RP-Select B for analysis in first instance of the water samples, because even though the run time necessary to complete the analysis is considerably shorter in the case of the LiChrospher 60 RP-Select B column (27 min), as compared to that of the LiChrospher 100 RP-18 column (42 min), it gives baseline separation of all analytes and, therefore, allows the use of both the UV and the MS detector. The LiChrospher 60 RP-Select B column, on the contrary, does not completely resolved all analytes. Diethylstilbestrol and levonorgestrel, and ethinylestradiol and estrone, partially coelute in this column,

Table 4
Recovery percentages obtained from the LC–DAD analysis of different distilled water sample volumes spiked at 10 μg/l with each analyte and extracted with a variety of SPE cartridges

	Recovery (%)								
	SPE: isolute ENV			SPE: LiChrolut EN			SPE: LiChrolut RP-18		
	Sample 250 ml	Sample 500 ml	Sample 1000 ml	Sample 250 ml	Sample 500 ml	Sample 1000 ml	Sample 250 ml	Sample 500 ml	Sample 1000 ml
Estriol	19.45	9.52	37.72	26.98	24.84	39.36	77.72	89.87	88.16
Estradiol	72.30	43.87	83.11	78.79	81.14	90.13	80.23	96.87	87.48
Norethindrone	100.61	100.27	99.06	101.31	100.17	102.88	95.89	91.58	96.31
Ethinyl estradiol	89.11	73.02	89.05	79.23	84.22	90.30	71.81	95.91	78.88
Estrone	97.03	67.17	97.76	92.25	90.51	99.87	91.72	97.66	100.44
Levonogestrel	100.49	109.96	96.96	111.72	105.82	104.15	95.97	91.58	100.58
Diethylstilbestrol	57.66	22.79	68.09	31.35	44.52	58.98	44.61	66.94	58.02
Progesterone	84.30	81.09	91.90	95.39	93.95	98.00	97.03	83.97	98.96
Mestranol	101.71	101.10	99.23	98.73	99.27	101.81	97.30	96.00	95.85
Ethynodiol diacetate	–	–	–	–	–	–	–	–	–

thus, making it difficult to use the UV detector. Standard solutions along with the samples were always included in the sequences in order to construct proper calibration curves.

The injection volume was 20 μl. Injection volumes greater than that lead to broader peaks and consequently to loss of resolution.

UV detection was performed at 197, 225, and 242 nm. Fig. 3 presents the chromatograms obtained from the analysis of a standard mixture containing 10 μg/l of each analyte at these wavelengths. At 225 nm, all analytes with the exception of ethynodiol diacetate exhibit some absorption. However, to improve selectivity and sensitivity, and to aid in identification through ratioing between peak intensities recorded at different wavelengths, UV detection was also performed at 197 nm, wavelength at which the group of estrogens shows characteristic absorption maxima; and at 242 nm, which is characteristic for the group of progestogens.

All steroid hormones as well as the synthetic analogues have in common the cyclopentanophenanthrene nucleus (see Fig. 1) [43]. With the exception of diethylstilbestrol, which is a non-steroidal compound, all analytes possess one methyl group (an ethyl group in the case of levonorgestrel) at position C-13, one or two substituents at position C-17, and an oxygen atom in the form of either a hydroxyl group or a carbonyl group, at C-3. However, estrogens and progestogens differ in their ring A structure, which is fully unsaturated (aromatic) in the former whereas in the later has only one alkene-

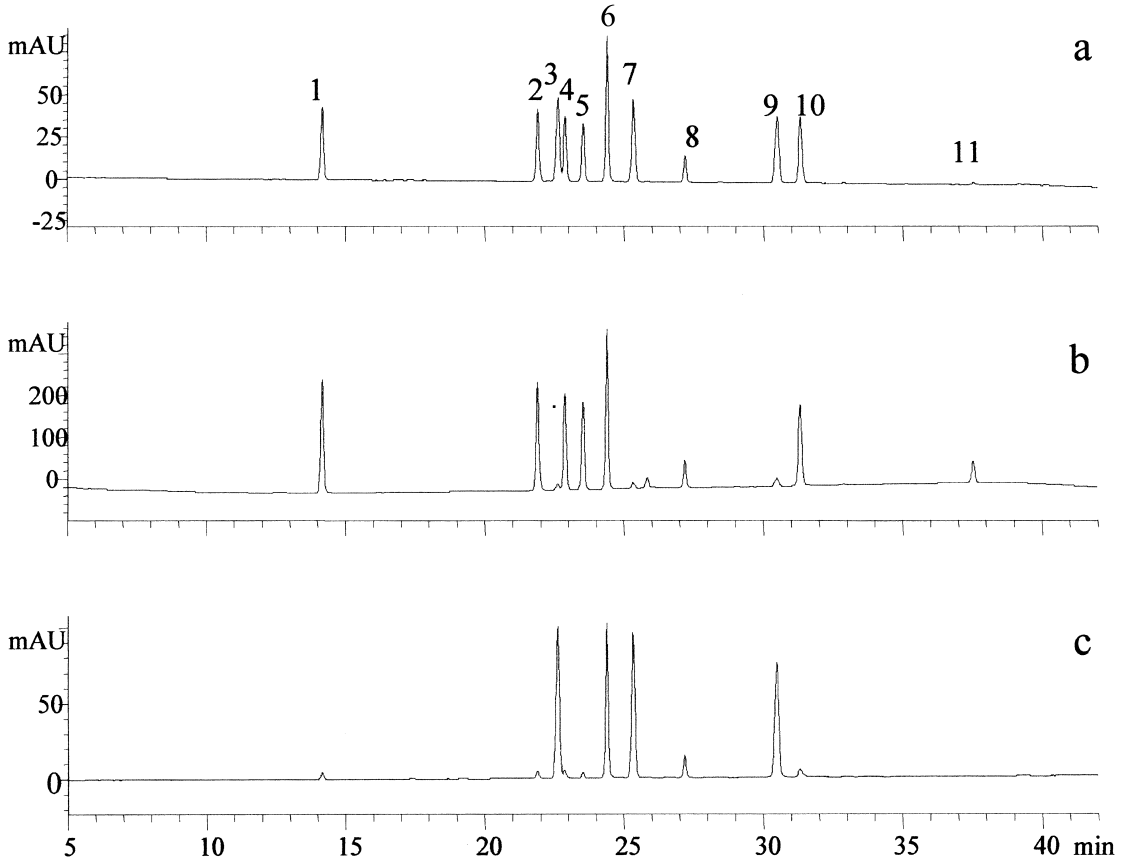


Fig. 3. Chromatograms obtained from the analysis of a standard mixture containing 10 μg/l of each analyte at wavelengths (a) 225, (b) 197, and (c) 242 nm. Column: LiChrospher 100 RP-18. Gradient elution: From 10% acetonitrile in water to 100% acetonitrile in 40 min. Flow rate: 1 ml/min. Peak identification: Estriol (1); estradiol (2); norethindrone (3); ethinyl estradiol (4); estrone (5); diethylstilbestrol (6); levonorgestrel (7); diethylstilbestrol isomer? (8); progesterone (9); mestranol (10); ethynodiol diacetate (11).

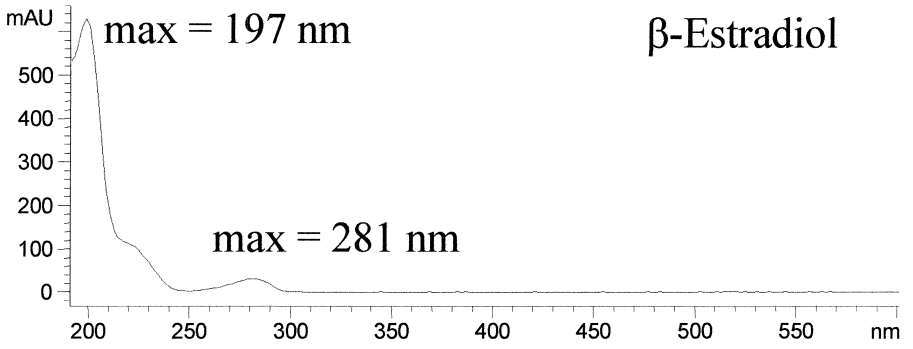


Fig. 4. UV spectrum of 17β-estradiol, representative of the UV spectrum characteristic of the estrogens group.

type double bond (C=C) at 4.5-position, and this difference is responsible for their distinctive UV spectra. Figs. 4 and 5 show characteristic UV spectra for both estrogens and progestogens.

The uv spectrum characteristic of the estrogens presents two maxima, one at approximately 199 nm and another at 281 nm, due to $\pi \rightarrow \pi^*$ transitions in the aromatic ring. On the other hand, the progestogens show a characteristic absorption maximum at approximately 242 nm which arises from $\pi \rightarrow \pi^*$ transitions in this case in the conjugated α,β-unsaturated ketone (C=C–C=O) at position 3 [44]. Ethynodiol diacetate, on the contrary, does not present either chromophore in its structure and as a consequence it hardly absorbs in the uv region.

For MS detection two interfaces—ESP and APCI—in both the positive and the negative ion mode were optimized for operation and subsequently intercompared for sensitivity and linearity of response. Optimization of the various parameters influencing the MS signal was carried out by LC and/or flow injection analysis (FIA) under full scan conditions (m/z 100 to 500). Table 2 shows the MS parameters resulting from such optimization.

Under these conditions, all compounds undergo very light fragmentation showing only one predominant ion. This predominant ion corresponds in the case of the estrogens, which are detected at acceptable concentrations only in the negative ion mode of operation with ESP, to the molecular ion. In the case of the progestogens, which can be detected in the positive ion mode with both the APCI and the ESP interfaces, the predominant ion corresponds to the molecular ion when the interface employed is APCI and to adducts of the analyte molecule with one sodium atom when the interface employed is ESP (see Table 3). Figs. 6 and 7 show characteristic mass spectra of both groups—estrogens and progestogens—of analytes.

MS conditions provoking light fragmentation and single predominant ions, instead of stronger conditions yielding more than one characteristic ion,

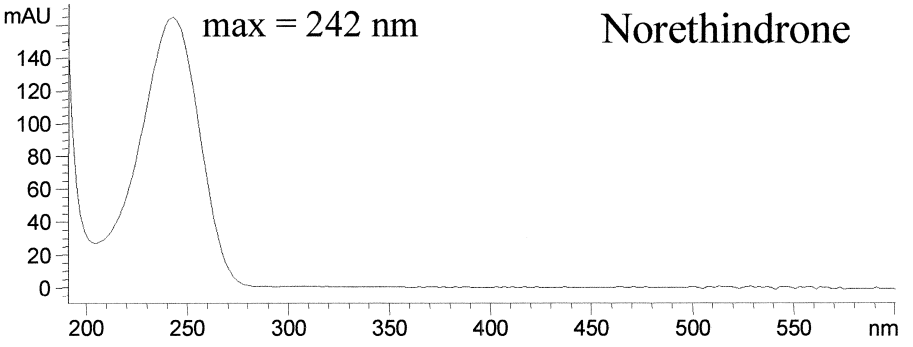


Fig. 5. UV spectrum of norethindrone, representative of the UV spectrum characteristic of the progestogens group.

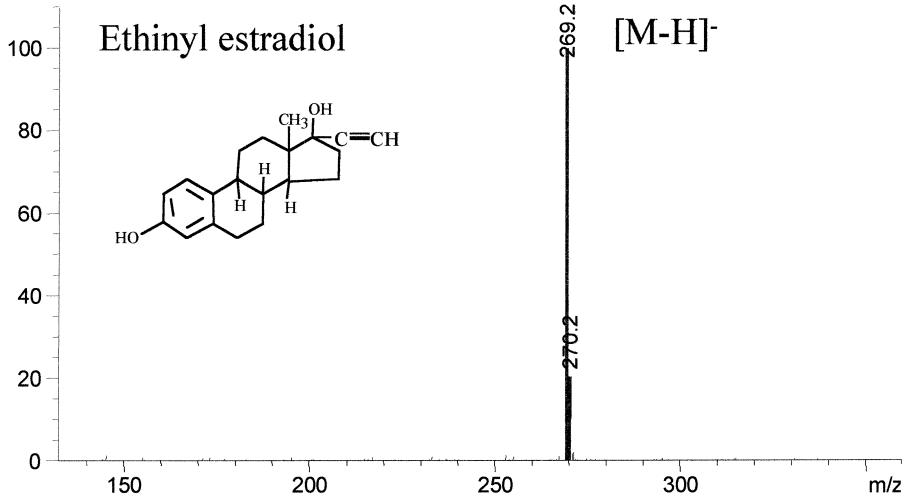


Fig. 6. MS spectrum of ethinyl estradiol, representative of the ESP(negative ion)-MS spectrum characteristic of the estrogens group.

were selected as optimum in order to get maximum, sufficient sensitivity as to enable the determination of the analytes at the very low concentrations at which they are present in the aquatic environment, and also because identification assessment could be done by other means: (1) From the DAD data as already mentioned, and (2) from the comparison of the retention times obtained with the two analytical columns, approach which is considered as more reliable than that based on the comparison of the MS signals obtained for two or more ions of a particular compound in the same chromatographic analysis.

The effects of mobile-phase additives on ionization efficiency were also evaluated. Modification of the acetonitrile–water mobile phase with methanol in various proportions, acetic acid 0.5%, or triethylamine 5 m*M*, did not improve significantly the MS signals.

Upon establishing the sensitivity and linearity associated to each interface, ESP was selected as first

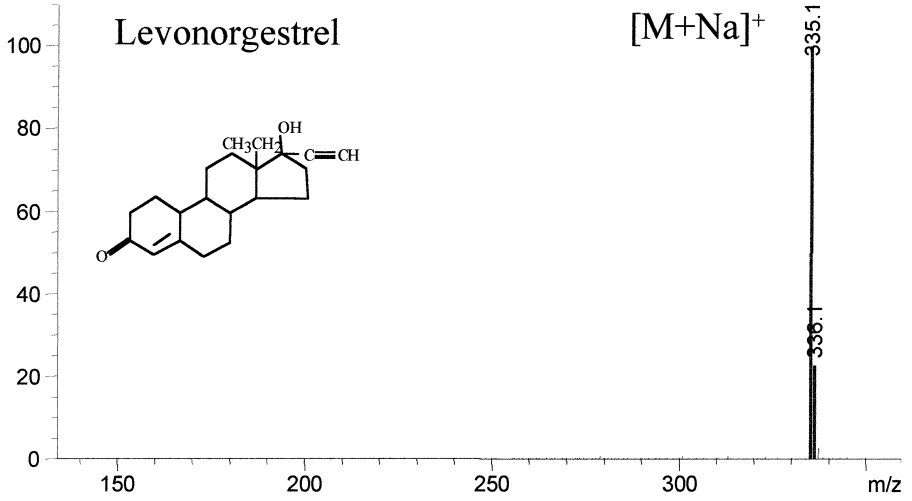


Fig. 7. MS spectrum of levonorgestrel, representative of the ESP(positive ion)–MS spectrum characteristic of the progestogens group.

option for the analysis of both estrogens and progestogens in water samples, and APCI in the positive ion mode of operation as second option for the analysis of progestogens.

3.2. Method performance

The method performance was evaluated by the determination of the linearity, sensitivity, selectivity, repeatability, and accuracy of the method.

For quantitation the external standard method was used in all instances. [$^2H_2$]17β-Estradiol was initially considered for its use as internal standard but finally discarded. The coelution with its homologue non-deuterated 17β-estradiol and the tight molecular mass difference (2 u) between these two compounds restrained its use as internal standard with neither detector (DAD or MS). The Hewlett-Packard LC–MS ChemStation software application was used to assist in the quantitation, based on peak areas, of standards and samples.

Five-point calibration curves were constructed using a least-square linear regression analysis from the injection of standard solutions of the mixture of all analytes at concentrations ranging from 1 to 100 μg/ml. When MS detection was accomplished in the positive ion mode of operation, with either ESP or APCI, seven-point calibration curves were constructed covering a wider range of concentrations (10 μg/ml to 25 ng/ml) as a consequence of its considerably better sensitivity. Table 5 lists the correlation coefficients (r^2) obtained for every analyte and detector. Good linearity was observed ($r^2 > 0.99$) except in the case of the MS detector when operating with the ESP interface in positive ion mode ($r^2 > 0.94$). An explanation for such low correlation coefficients could be in the likely instability of the adducts formed between the analyte molecule and the sodium atom, which were used for quantitation in this case.

Detection limits (DLs) (see Table 5) were experimentally estimated from the injection of standard solutions serially diluted until the signal-to-noise ratio (S/N) for any single analyte reached a value of three. DLs fell between 50 and 500 ng/l for DAD, between 2 and 500 ng/l for ESP–MS, and between 20 and 5000 ng/l for APCI–MS. As it can be seen, mestranol is not detected by any of the MS interfaces and modes tested and, therefore, its simultaneous determination with the rest of the analytes considered in this study requires the use of the diode array detector in series with the MS detector.

Since no certified reference materials were available, the overall method repeatability and accuracy was determined from the analysis of six replicates of distilled water (0.5 l) spiked with a standard mixture of the analytes at 10 μg/l (see Table 6). Satisfactory recoveries (83%) were obtained for all compounds except diethylstilbestrol (56%), and ethynodiol diacetate. The low recovery percentage obtained for

Table 5
Calibration curve correlation coefficients (r^2) and detection limits (DLs) (in ng/l) obtained for each analyte and detector

Compound	DAD					ESP		APCI	
	197/242 nm			225 nm					
	λ (nm)	r^2	DL	r^2	DL	r^2	DL	r^2	DL
Estriol	197	0.9996	50	1.0000	100	0.9945	50	–	5000
Estradiol	197	0.9996	50	0.9999[a]	100	0.9979	250	–	3000
Ethinyl estradiol	197	0.9998	50	0.9999	100	0.9975	500	–	3000
Estrone	197	0.9998	50	0.9999	100	0.9988	100	–	3000
Diethylstilbestrol	242	0.9995	50	0.9996	100	0.9982	25	–	2000
Mestranol	197	0.9998	50	1.0000	100	ND	ND	ND	ND
Norethindrone	242	1.0000	50	0.9999	100	0.9484	2	0.9999	20
Levonogestrel	242	0.9995	50	0.9989	100	0.9495	2	0.9995	20
Progesterone	242	1.0000	100	1.0000	100	0.9474	2	0.9997	20
Ethynodiol diacetate	197	0.9970	500	ND	ND	0.9493	10	0.9977	100

[a] ND: Not detected.

Table 6
Accuracy and repeatability data from the LC–DAD–MS analysis of six replicates of distilled water (0.5 l) spiked at 10 μg/l with each analyte

	ESP			UV		
	Ion mode	Recovery (%)	RSD (%)	λ (nm)	Recovery (%)	RSD (%)
Estriol	–	84.91	16.58	197	89.87	17.31
Estradiol	–	92.71	17.82	197	96.87	10.89
Norethindrone	+	104.09	13.75	242	91.58	14.89
Ethinyl estradiol	–	93.75	18.72	197	95.91	11.49
Estrone	–	92.81	19.36	197	97.66	11.70
Diethylstilbestrol	–	56.64	22.68	197	66.94	17.76
Levonogestrel	+	112.71	18.42	242	91.58	14.99
Progesterone	+	112.86	25.49	242	83.97	14.74
Mestranol	ND[a]	ND	ND	197	96.00	11.78

[a] ND: Not detected.

diethylstilbestrol is probably not a consequence of the extraction procedure but the result of some kind of equilibrium between two different isomeric forms of the compound, yet not determined, as reveals the presence of two peaks with the same mass spectrum at different retention times and with intensities that increase and decrease in approximately reversed proportion. Ethynodiol diacetate, as stated in the sample preparation part, is not recovered at all from the SPE cartridge.

The overall method repeatability was satisfactory although, as it could be expected, better with the UV detector (RSD<18%) than with the MS detector (RSD<25%).

3.3. Environmental samples

Figs. 8 and 9 show the LC–ESP–MS chromatograms obtained under SIM conditions in the positive and the negative ion mode, respectively, from the analysis of various types of water. As it can be seen, the method is highly selective for the analysis of this kind of compounds in most environmental waters, including drinking water, surface water, and STP effluents. This selectivity added to the sensitivity achieved with the MS detector allows for the quantitation of the progestogens at the low ng/l levels likely to be found in the real samples, and for the quantitation of the estrogens at slightly higher concentrations (ng/l), with specific useful ranges varying for every single compound between the detection limit and the upper limit of the corresponding calibration curve. Waste-waters, or more precisely, STP influents give comparatively more complex chromatograms that natural or treated waters, and the applicability of the method to the determination of the group of estrogens in this case is limited.

4. Conclusions

While findings correlating environmental estrogens and progestogens with adverse human health effects remain one of the main focus of scientific debate and investigation with regards to the field of EDCs, the development of appropriate analytical methods for determining this type of compounds in water is, likewise, a very important task in order to assess human exposure to these environmental pollutants. In the present work, a sensitive and selective method, based on off-line SPE of the water sample followed by LC–DAD–MS analysis, has been developed and validated. It allows the unequivocal determination of the most common and biologically active natural and synthetic estrogens and progestogens in water at the ng/l level with satisfactory precision (RSD<25%) and accuracy (recovery>83%) for most analytes. The method here described is currently being applied to the evaluation of the estrogenic and progestogenic content of various types of water from the area of Catalonia (NE Spain) and it will be the subject of future publications.

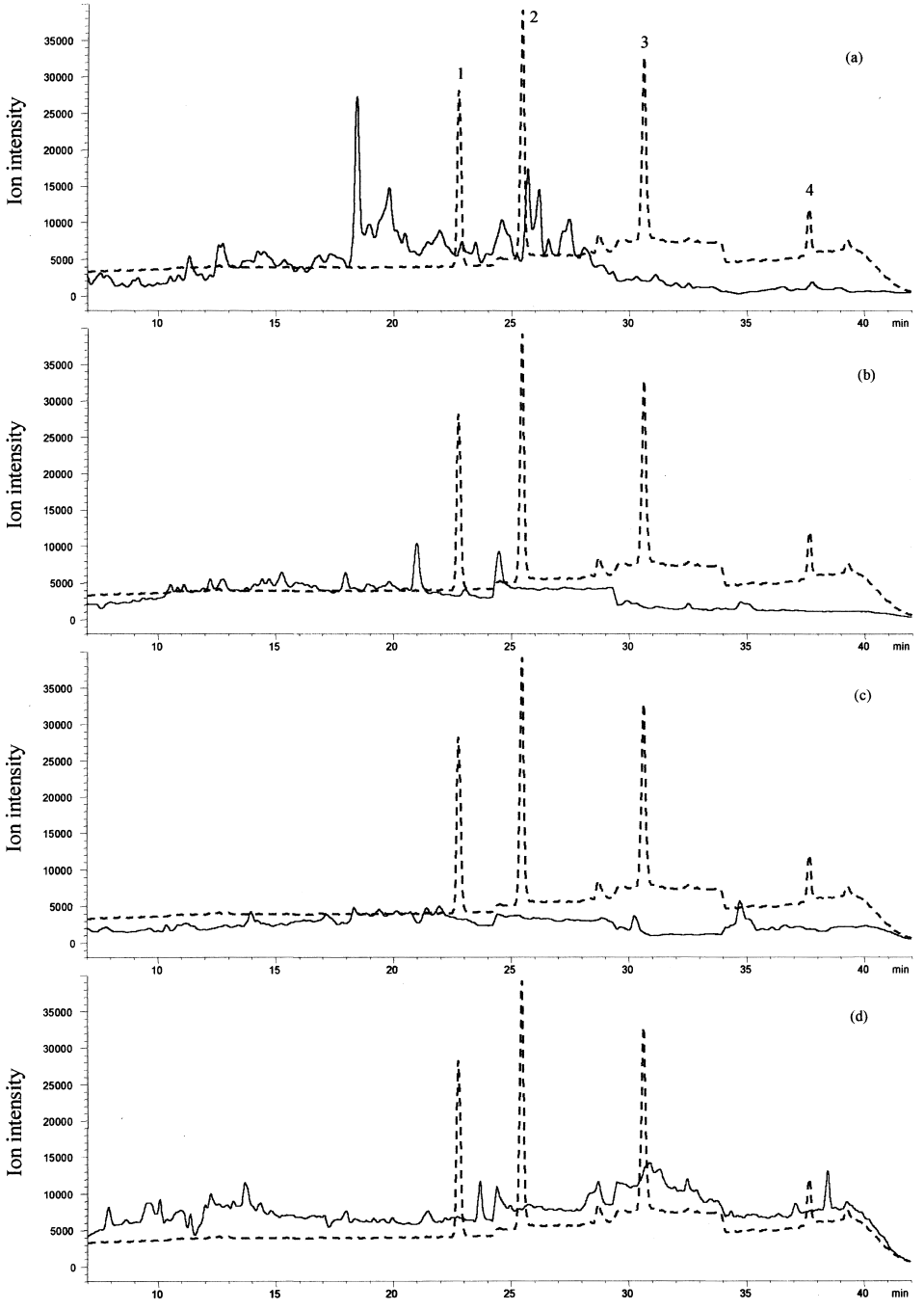


Fig. 8. LC–ESP–MS chromatogram of the analysis in the positive ion mode of various types of water (———) (a) SWTP influent, (b) SWTP effluent, (c) highly polluted river water, (d) drinking water; and their comparison with a standard mixture containing each of the analytes at 25 ng/ml in methanol (equivalent to 25 ng/l water considering the method concentration factor of 10^3) (-----). Column: LiChrospher 100 RP-18. Gradient elution: From 10% acetonitrile in water to 100% acetonitrile in 40 min. Flow rate: 1 ml/min. Peak identification: Norethindrone (1); levonorgestrel (2); progesterone (3), and ethynodiol diacetate (4).

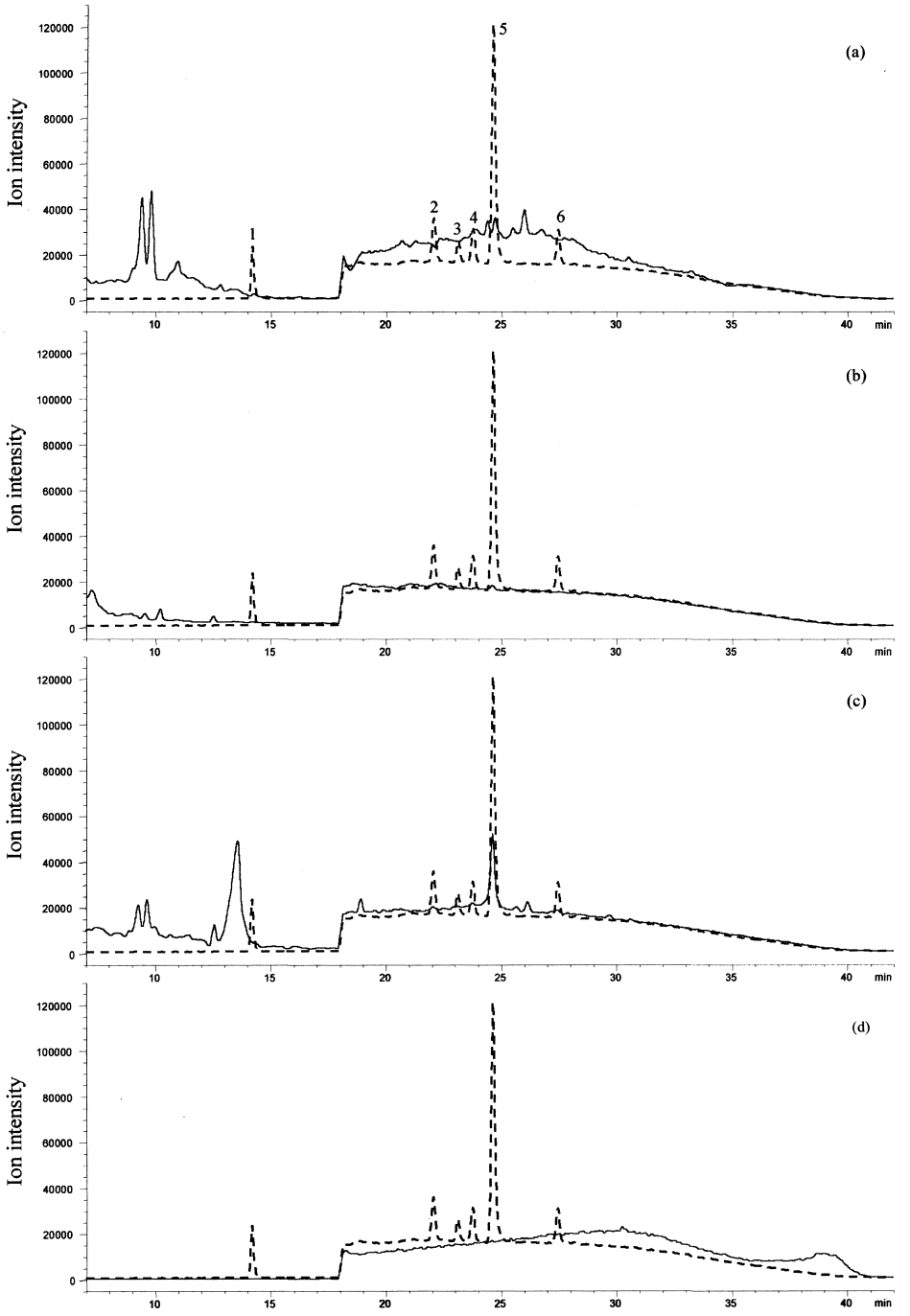


Fig. 9. LC–ESP–MS chromatogram of the analysis in the negative ion mode of various types of water (———) (a) SWTP influent, (b) SWTP effluent, (c) highly polluted river water, (d) drinking water; and their comparison with a standard mixture containing each of the analytes at 1 μg/ml in methanol (equivalent to 1 μg/l water considering the method concentration factor of 10^3) (-----). Column: LiChrospher 100 RP-18. Gradient elution: From 10% acetonitrile in water to 100% acetonitrile in 40 min. Flow rate: 1 ml/min. Peak identification: Estriol (1); estradiol (2); ethinyl estradiol (3); estrone (4); diethylstilbestrol (5), and diethylstilbestrol isomer? (5).

Acknowledgements

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