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Use of solid-phase extraction in various of its modalities for sample preparation in the determination of estrogens and progestogens in sediment and water

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Abstract

The environmental analysis of estrogens and progestogens at physiologically active concentrations (low ng/l range) requires the use of very sensitive and selective methods, which, in most cases, make necessary an extraction/purification step. In this study, various procedures for the determination of several estrogens (estriol, estradiol, ethynyl estradiol, estrone, and diethylstilbestrol) and progestogens (progesterone, norethindrone, and levonorgestrel) in environmental matrices, including water and river sediment, are described. In all procedures, final analysis of the target compounds is performed by reversed-phase liquid chromatography–diode array detection–mass spectrometry, whereas sample preparation always includes a solid-phase extraction (SPE) step. For this SPE step, various types of sorbents, protocols, and devices have been used, and their respective advantages and disadvantages are discussed. For the off-line SPE of estrogens and progestogens from water samples, a syringe type cartridge LiChrolut RP-18 (500 mg) was selected out of two other sorbents — LiChrolut EN (200 mg) and Isolut ENV (500 mg) — for use with the automated sample preparation instrument ASPEC XL. For the on-line SPE and analysis of water samples the 10 mm×2 mm I.D. HySphere-Resin-GP cartridge, was preferred to the C_{18} Baker, the PLRP-S, and the Oasis HLB, for use with the Prospekt system. A completely manual protocol based on the use of Sep-Pak C_{18} Plus cartridges was developed for purification of sediment extracts. All procedures were shown to be linear over a wide range of concentration, exhibited satisfactory repeatability and accuracy, and reached limits of detection usually in the low ng/l and ng/g range. Comparatively, the on-line method was shown to be advantageous in terms of automation and general method performance. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Solid phase extraction; Sediments; Water analysis; Environmental analysis; Estrogens; Progestogens; Steroids

1. Introduction

The presence of female sex hormones, such as 17-β estradiol and estrone, and of synthetic steroids, such as ethynyl estradiol, a chemical largely used as a contraceptive, in the aquatic environment, has raised great concern. These potent estrogenic compounds have been shown to induce estrogenic responses in fish at concentrations in water (0.1–1 ng/l) [1–3] lower than those commonly detected in the environment (ng/l), while the potential consequences to humans remain yet uncertain.

Both natural and synthetic estrogens and progestogens are excreted in the urine of mammalians, and a small proportion in the feces, and via the effluent of sewage treatment plants (STPs) or through run-off

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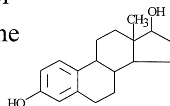
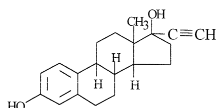
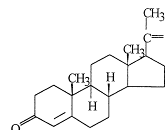
- ESTROGENS
  - Natural hormones:
    - Estradiol
    - Estriol
    - Estrone



  - Synthetic compounds:
    - Ethynyl estradiol
    - Diethylstilbestrol



- PROGESTOGENS
  - Natural hormones:
    - Progesterone



  - Synthetic compounds
    - Norethindrone
    - Levonorgestrel

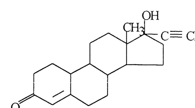


Fig. 1. Target analytes and representative chemical structures (corresponding to the compounds underlined).

of sewage sludge used for agriculture, enter waterways. They can lead to reproductive and developmental alterations in aquatic organisms, such as feminization or hermaphroditism.

The analysis of these compounds in the environment constitutes a difficult task, first, because of the complexity of the environmental matrices, and second, because of their very low, physiologically active, environmental concentrations. Thus, very sensitive and selective analytical methodologies, which in general translate on long and laborious procedures, are needed for their accurate determination.

A typical analytical procedure includes, within the sample preparation, various steps, such as filtration, extraction, purification, hydrolysis, derivatization, and evaporation. For analysis, immunoassays, and to a greater extent gas chromatography–mass spectrometry, have been the techniques most commonly employed, whereas liquid chromatography–mass spectrometry has gained in popularity in the last few years [4]. The advantage of using liquid chromatography (LC) is that the enzymatic hydrolysis step, required for the immunoassay analysis of both conjugated (glucuronides, sulfates, etc.) and unconjugated estrogens and progestogens, and the derivatization step that normally precedes a subsequent GC–MS analysis, can be obviated.

Of the various steps in a sample preparation procedure, the extraction/purification step, which is present in almost all the analytical procedures described in the literature, is the most critical. This step can be performed, depending on the kind of sample, by different means, but the method most commonly employed nowadays for this purpose is solid-phase extraction (SPE). As it is well known, for SPE, a wide variety of sorbents, sorbent cartridges and devices are commercially available. Their selection depends on a series of factors that include kind of sample, the selectivity and sensitivity required, cost, etc. The scope of this study is to evaluate, through the description of various methodologies developed for the analysis of estrogens and progestogens in various types of environmental samples, the applicability of the SPE to this type of analysis and the advantages and disadvantages associated with the use of some of these commercially available sorbents, cartridges and devices.

As target compounds (see Fig. 1) we selected, based on their abundance in the human body, their estrogenic potency, and the extent of their use in contraceptive pills, the natural estrogen estradiol and its main metabolites estriol and estrone, the synthetic estrogens ethynyl estradiol and diethylstilbestrol, the natural hormone progesterone, and the synthetic progestogens, norethindrone and levonorgestrel.

2. Experimental

2.1. Chemicals

Pure standards of both natural and synthetic estrogens and progestogens (Fig. 1) 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. Standards for on-line analysis were prepared by subsequent spiking of LC-grade water with the standard mixtures in methanol and the final standard aqueous solutions did not contain more than 0.1% of methanol.

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

2.2. Sample preparation

For analysis of water samples two different procedures, one on-line and one off-line, were developed. In the case of the river sediment, the various steps integrating the sample preparation were performed off-line with the final analysis by LC–diode array detection (DAD)–MS.

2.2.1. Water samples off-line

In the off-line method, described elsewhere in more detail [5], water samples were filtered through 0.45 μm glass fiber filters and subsequently extracted onto LiChrolut RP-18 cartridges (500 mg, 3 ml) with an automated sample processor ASPEC XL fitted with a 817 switching valve and an external 306 LC pump from Gilson (Villiers-le-bel, France). Conditioning of the cartridges was performed with 7 ml of acetonitrile, 5 ml of methanol, and 5 ml of LC-grade water at a flow-rate of 3 ml/min. After loading of the sample (1000 ml at 5 ml/min) and subsequent washing with 5 ml of reagent water at 5 ml/min, the cartridges were dried with a Baker LSE 12G apparatus (J.T. Baker, Deventer, Netherlands) connected to a vacuum system and put back again on the ASPEC XL apparatus for elution with 2×4 ml of acetonitrile. The so-obtained extracts were then blown down to dryness under nitrogen and reconstituted in methanol to a final volume of 0.5 ml for further LC–DAD–MS analysis.

2.2.2. Water samples on-line

In the on-line method, optimized from a previously described procedure [6], fully automated on-line trace enrichment of the water samples was performed on 10×2 mm HySphere-Resin-GP cartridges (Spark Holland, Netherlands) with an automated sample preparation system (Prospekt, Spark Holland) consisting of a cartridge exchange module, a solvent delivery unit (SDU) and a low-pressure six-port valve connected on-line to the chromatographic system. The samples (0.2–1 l), previously filtered as above, were passed at 6 ml/min through the cartridges previously conditioned with 4 ml of acetonitrile and 4 ml of LC-grade water (flow-rate 2 ml/min). After sample loading and prior to elution, 4 ml of LC-grade water were passed at a flow-rate of 6 ml/min to complete transfer of the sample and wash the cartridge. Elution of the trapped compounds to the LC column was carried out with the chromatographic mobile phase.

2.2.3. Sediment samples off-line

Freeze-dried river sediment samples (5 g) were extracted by ultrasonics with 25+15+15 ml (5 min each) of a mixture methanol–acetone (1:1). After centrifugation for 5 min at 4000 rpm, the liquid phases obtained from each three extraction steps were combined, concentrated to dryness by rotary evaporation, and re-dissolved in 2 ml of the mixture methanol–acetone (1:1) plus 18 ml of reagent water. The so-obtained extracts were then purified by SPE using Sep-Pak C_{18} Plus cartridges (Waters, Milford, MA, USA) conventionally conditioned. The eluates resulting from passing 8 ml of acetonitrile through the cartridges loaded with the sample were then blown down to dryness under nitrogen and finally reconstituted in 0.5 ml of methanol for subsequent LC–DAD–MS analysis.

2.3. LC–DAD–MS analysis

Detail information about the chromatographic and detection conditions is described elsewhere [5,6]. Briefly, the chromatographic system consisted of an

HP1100 autosampler, with the volume injection set to 20 μl, and an HP1100 LC pump connected in series with a DAD system model HP1100 and a HP1100 mass-selective detector with atmospheric pressure ionization (electrospray ionization, ESI), all from Hewlett-Packard (Palo Alto, CA, USA). In the on-line system the Prospekt instrument replaced and acted as the autosampler. Separation was achieved 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). A gradient elution from 10 to 100% acetonitrile in water in 40 min at a flow-rate of 1 ml/min was used as mobile phase.

UV detection in series with MS was performed with the purpose of aiding identification and evidencing eventual MS signal suppressions. Chromatograms were recorded at 197 nm for quantitation of the estrogens, at 242 nm for quantitation of the progestogens, and at 225 nm, wavelength at which all analytes exhibit some absorption. UV spectra from 190 to 600 nm were also registered.

MS detection was performed under time-scheduled selected ion monitoring conditions, by using an electrospray interface operating in the negative ion (NI) mode for determination of estrogens and in the positive ion (PI) mode for determination of progestogens. Molecular ions $[M-H]^-$ and adducts $[M+Na]^+$ of the analyte molecule with one sodium atom were registered in the NI and the PI mode, respectively, under the following MS conditions: nebulizer pressure, 55 p.s.i.; drying gas flow, 13 l/min; drying gas temperature, 300 and 350°C (for NI and PI, respectively); capillary voltage, 3500 and 6000 V (for NI and PI, respectively); and fragmentor, 110 and 90 V (for NI and PI, respectively) (1 p.s.i.= 6894.76 Pa).

3. Results and discussion

3.1. Sample preparation

A series of sample pre-treatments, other than the solid-phase extraction step, were carried out within the sample preparation protocols. In this respect, it should be mentioned that the filtration step [7,8], which is performed only in the case of water samples with high suspended matter content, and the concentrations steps (rotary evaporation, stream of nitrogen, etc.) [8], have been shown not to lead to significant losses of the analytes.

For the SPE or purification step, performed at different stages in all three protocols described, different types of cartridges, sorbents, and devices were employed.

3.1.1. SPE sorbents

SPE or purification of estrogens and progestogens from environmental matrices has been mostly performed by means of octadecyl (C_{18}) silica bonded phases, polymeric sorbents, and combinations of them [9,10], although the use of graphitized carbon black has also been reported occasionally in the literature [11–13].

For the off-line SPE of water samples [5], three different sorbents packed in disposable syringe type cartridges were evaluated: LiChrolut EN (200 mg), LiChrolut RP-18 (500 mg), both from Merck, and Isolut ENV from International Sorbent Technology (Cambridge, UK). All three sorbents probed similar, good extraction capacity and elution efficiency for most of the target analytes (Table 1). However, LiChrolut RP-18 was the only phase capable of satisfactorily extracting estriol. In the other sorbents tested, the estriol experimented, regardless of the sample volume, breakthrough, as a consequence of its capacity factor.

For the on-line SPE and analysis of the same analytes in water [6], four different 10 mm×2 mm I.D. disposable trace enrichment cartridges were evaluated: the octadecyl-bonded silica cartridge C_{18} Baker (40 μm) (J.T. Baker) and the polymeric cartridges PLRP-S (15–25 μm) (Polymer Labs, Church Stretton, UK), HySphere-Resin-GP (5–15 μm) (Spark Holland), and Oasis HLB (30–60 μm) (Waters). Contrary to the results previously observed when evaluating the extraction efficiency of the sorbents in the off-line approach, the C_{18} cartridge was the only phase exhibiting poor extraction efficiency towards estriol for a sample volume of 200 ml, whereas the rest of the compounds investigated were satisfactorily extracted and to a similar extent by all four cartridges evaluated (Table 2). However, for extraction of larger volumes of sample (1000 ml) the HySphere-Resin-GP cartridge was finally pre-

Table 1
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

	SPE cartridge								
	Isolute ENV			LiChrolut EN			LiChrolut RP-18		
Sample volume (ml)	250	500	1000	250	500	1000	250	500	1000
Estriol	19	10	38	27	25	39	78	90	88
Estradiol	72	44	83	79	81	90	80	97	87
Norethindrone	101	100	99	101	100	103	96	92	96
Ethynyl estradiol	89	73	89	79	84	90	72	96	79
Estrone	97	67	98	92	91	100	92	98	100
Levonogestrel	100	110	97	112	106	104	96	92	101
Diethylstilbestrol	58	23	68	31	45	59	45	67	58
Progesterone	84	81	92	95	94	98	97	84	99

ferred to the other polymeric phases, because it gave a comparatively better recovery than the PLRP-S cartridge, and because, unlike the Oasis cartridge, it did not provoke band-broadening.

For clean-up of the extract obtained from the sediment sample, various sorbents packed in Sep-Pak Plus cartridges (Waters), including C_{18}, alumina B, silica, and CN, and combinations of them (C_{18}+ NH_2), were evaluated. The C_{18} sorbent was revealed as the most adequate, in terms of recovery and selectivity, for the overall purification of the target compounds (unpublished observations).

3.1.2. SPE cartridges and devices

As already mentioned, syringe-type cartridges were used for the off-line SPE of water samples carried out with the aid of the ASPEC XL (automated sample preparation with extraction columns) instrument. This instrument performs the cartridge conditioning, the sample loading, and the subsequent washing of the cartridge in an automated way, after which the cartridges are removed from the instrument for drying with air or nitrogen under positive or negative pressure, and put back again in the instrument for elution. All these steps can also be performed with the aid of other instruments such as the Baker LSE 12G, used in the methodologies here described solely for drying of the cartridges. The advantages of the ASPEC XL over the Baker LSE 12G are its higher degree of automation, since a larger number of steps can be carried out without the operator's intervention, and that both the flow-rate and the volume of samples and solvents can be more accurately controlled. In turn, the Baker LSE 12G

Table 2
Comparison of the recovery percentages obtained from the analysis (n=3) of various sample volumes of spiked LC-grade water extracted with a variety of SPE cartridges

	50 ml sample volume, 10 μg/l spiking level				100 ml sample volume, 10 μg/l spiking level				200 ml sample volume 1 μg/l spiking level		
	RP-18	Oasis	HySphere	PLRP-S	RP-18	Oasis	HySphere	PLRP-S	Oasis	HySphere	PLRP-S
Estriol	87	96	94	95	76	97	94	95	99	98	94
Estradiol	97	94	93	96	100	98	98	100	97	95	94
Norethindrone	99	98	95	98	101	100	100	101	97	96	96
Ethynyl estradiol	92	90	89	91	97	95	90	93	98	98	98
Estrone	97	101	93	95	98	98	95	98	101	97	97
Diethylstilbestrol	70	71	63	68	83	81	67	79	87	66	78
Levonorgestrel	95	101	91	93	97	102	96	97	98	95	94
Progesterone	94	93	92	92	98	96	95	95	94	92	90

apparatus is more advantageous, compared to the ASPEC XL, in that it is considerably cheaper, various samples (up to 12 in this design) can be simultaneously processed (not one by one as in the ASPEC XL), and it is also more versatile, because of its physical design. Thus, the existence of no physical impediments to set various cartridges in series on top of the vacuum cage permits, for example, coupling the sorbent cartridge used for extraction with a filter holder to perform both the filtration and the extraction of the sample in a single step, or coupling the extraction cartridge with a sodium sulfate column to obtain water-free eluates. An additional advantage, in the case of both instruments, is the wide variety of syringe-type cartridges commercially available in both glass and plastic holders and for a great number of sorbents, sorbent amounts and volume capacities.

The other procedure, developed for the on-line extraction and analysis of estrogens and progestogens in water, made use of the Prospekt system. This system analyses up to 16 samples in a completely automated, unattended way, with the corresponding operator's time and labor saving, and its simplicity obviates the need for highly qualified or experienced staff. This type of approach is also very advantageous, in comparison with off-line methodologies, in terms of sensitivity, because the whole sample instead of an aliquot of the final extract, as in off-line protocols, is transferred to the chromatographic system. Also, in terms of reproducibility and accuracy, because the sample manipulation is reduced to the filtration step or, in the case of waters with low suspended matter content, completely avoided. With regards to the extraction columns, a wide variety of sorbents packed in two different dimension cartridges (10× 2 and 10× 3 mm, I.D.) are also commercially available. Disadvantages of the on-line approach, as compared to off-line methodologies, are the limited possibility of combining different cartridge sorbents, mainly because of the restrictions imposed by the chromatographic mobile phase, and the unavailability of a final extract for parallel determinations.

A quite different approach, at least in terms of automation, was followed for the purification step carried out with the sediment samples after ultrasonic extraction. In this case, whilst a completely automated procedure, from extraction to analysis, is not feasible from the instrumental point of view, the rather small volume of extract to be processed (20 ml) in the purification step does not merit the employment of expensive, robotized instrumentation. Therefore, for this approach, Sep-Pak Plus cartridges with the single requirement of a regular syringe were used. The advantage of these completely manual protocols, in addition to the low cost and simplicity of instrumentation, is the versatility, already pointed out for the syringe-type cartridges, resulting from the possibility of connecting various columns with different extraction sorbents and materials. The general disadvantage, although irrelevant here, is that only reduced volumes are normally processed because extraction of, for instance, one liter of water would obviously take too much time for the operator.

3.2. Methods performance comparison

Table 3 summarizes several parameters indicative of the analytical performance of the various methodologies described, relative to the use of the mass spectrometric detector. As it has been already commented, the on-line methodology, although less versatile than the off-line protocols, provides, in addition to fully automation, better accuracy and repeatability. Thus, very good relative standard deviations (lower than 3%) and recovery percentages (between 96 and 112%) were obtained, when the MS detector had not been yet integrated in the system, from the on-line LC–DAD analysis of six replicates of spiked reagent water (200 ml) [6]. The slightly worse accuracy and repeatability results reported in Table 3, calculated with the MS detector from the analysis of six replicates of 1000 ml spiked reagent water by the on-line LC–DAD–MS procedure described here is, in fact, attributed to an inaccurate, at the time of analysis, dispensing of the sample volume by the solvent delivery unit coupled to the Prospekt, and not to the mass spectrometric detector or to the higher sample volume processed. The linearity of the calibration curves constructed from both the injection of standard solutions and the analysis of spiked samples was good in all three procedures, with correlations coefficients always higher than 0.99, but comparatively better in the on-line protocol due to the minimal sample manipu-

Table 3
Methods performance comparison

	ASPEC XL, LiChrolut RP-18, water sample	Prospekt, HySphere-Resin-GP, water sample	Manual, Sep-Pak C_{18} Plus, sediment sample
Repeatability (RSD, %)	<25	<18 (<3)[a]	<19
Accuracy (% recovery)	57–113	77–106 (96–112)[a]	71–103
Spiking concentration[b]	10 μg/l	50 ng/l (10 μg/l)[a]	1000 ng/g
Linearity (r^2)	>0.99	>0.99	>0.99
Calibration range	LOD — 10 μg/l	1–1000 ng/l	LOD — 1000 ng/g
Sensitivity (LOD)	1–20 ng/l	<1 ng/l	0.5–5 ng/g
Automation	Partial	Complete	No
Instrumentation	High	High	No
Versatility	Medium	Low	High

[a] Values in parenthesis calculated with the DAD detector and the PLRP-S cartridge.
[b] Used in the repeatability and accuracy studies ($n=6$).

lation. The sensitivity is perhaps the most important parameter for this particular analysis. The instrumental detection limits, experimentally estimated from the injection (20 μl) of serially diluted standard solutions until the signal-to-noise ratio (S/N) reached a value of three, fell between 0.4 and 10 ng/ml. The corresponding detection limits (LODs), calculated for each analyte and matrix by applying the appropriate off-line method concentration factor, gave values ranging between 0.2 and 5 ng/l water (concentration factor 2000), and between 0.04 and 1 ng/g sediment (concentration factor 10). A similar kind of approach for the on-line analysis of water yielded sensitivities 10 to 50 times better than the off-line protocol, as described here. The method detection limits shown in Table 3 and experimentally estimated from the analysis of spiked water and sediment samples, were, as expected, slightly higher than those just reported because of the matrix effect, but the comparatively better sensitivity of the on-line approach is evident.

3.3. Field studies

As a part of various environmental monitoring programs conducted for both determining the degree of pollution by endocrine disrupting compounds in rivers from the Catalonian area (NE of Spain), and establishing potential correlations between the observed chemical burden and the physiological alterations detected in fish, the off-line procedures described here were applied to the determination of the target estrogens and progestogens in samples of water and sediment [14–16].

These studies revealed the presence of both natural sex hormones and related synthetic chemicals in sewage treatment plant influents and effluents, and receiving river waters and sediments, at concentrations usually in the low ng/l water and ng/g sediment range. These surveys also demonstrated estriol, estrone, and progesterone as the most ubiquitous compounds of the various investigated. Likewise, certain relationships could be hypothesized between the environmental concentration of some of the target analytes in river water and sediments, but particularly between the estriol and the estrone concentration in water, and the vitellogenin induction observed in male carps captured at the same sampling sites. Fig. 2 shows the selected ion monitoring ($[M-H]^-$ 295) LC–ESI–MS chromatogram obtained from the off-line analysis of a river sediment where a considerably high concentration of ethynyl estradiol (22.8 ng/g)was observed; coinciding with an also high occurrence of intersexuality and vitellogenin induction. Studies carried out over the time also allowed us to elucidate seasonal variations in the environmental levels of these potentially harmful compounds, as well as varying removal efficiencies in the STPs monitored.

4. Conclusions

The widespread use of solid-phase extraction for

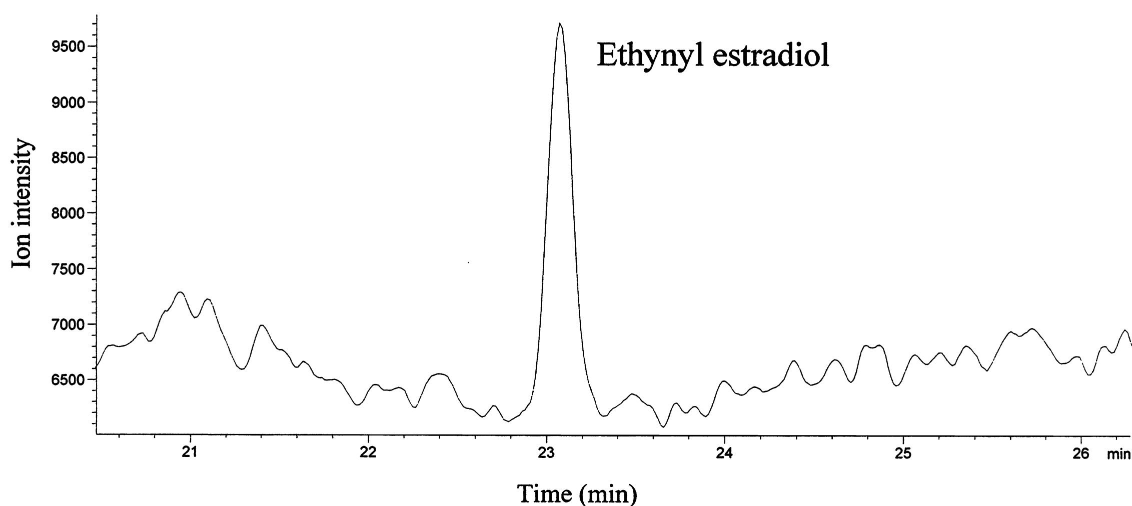


Fig. 2. Selected ion monitoring (*m/z* 295) LC–ESI–MS chromatogram obtained from the off-line analysis of a river sediment sample for ethynyl estradiol determination.

sample preparation in environmental analysis has led to the development and commercialization of multiple devices that differ mostly in the degree of automation and cost, and for which a great variety of cartridges and sorbents have been prepared. Examples of the application of many of these systems, with different degrees of automation, to the extraction and analysis of estrogens and progestogens in environmental matrices have allowed the study of their associated advantages and disadvantages. Thus, the completely automated on-line methodology here described has proven a very advantageous technique yielding better repeatability, accuracy, linearity, and sensitivity than the off-line protocols presented, in addition to automation and speed. The limiting factor of this kind of approach is the elevated cost and also the limited possibility of combining different extraction materials. Partially automatized methodologies, such as that described for the off-line analysis of water, have also a number of advantages that include time-saving, versatility on coupling different cartridges, availability of the final extracts, and different cost, commercially available, instrumentation. On the other hand, completely manual protocols are presented as the best option, from a practical and an economical point of view, for processing small volumes of samples or extracts, such as those processed in the case of the sediment samples. Finally, the introduction of new SPE materials, currently under development in the field of molecular imprinting polymers and immunoaffinity sorbents, and of more selective analytical techniques such as tandem mass spectrometry coupled to LC, is expected to greatly improve the performance of the current methodologies.

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