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Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by fully automated on-line solid-phase extraction–liquid chromatography–diode array detection

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Abstract

In this study, a procedure for the simultaneous determination in water of six estrogens (estradiol, estriol, estrone, ethynyl estradiol, mestranol, and diethylstilbestrol) and three progestogens (progesterone, norethindrone, and levonorgestrel), selected based on their abundance in the human body, their estrogenic potency, and the extent of their use in contraceptive pills, was developed. The procedure, based on the on-line solid-phase extraction (SPE) of the water sample and subsequent analysis by liquid chromatography/diode array detection (LC/DAD), allows for the monitoring of up to 16 samples in a completely automated, unattended way. The SPE experimental conditions were optimized and the polymeric cartridge PLRP-S selected out of four different cartridges evaluated. The chromatographic separation was carried out on a LiChrospher 100 RP-18 and detection was performed at 200, 225, and 240 nm. The applicability of the method to the analysis of various environmental water samples, including drinking water, groundwater, surface water and sewage treatment plant effluents, was evaluated. Method detection limits were in the range 10–20 ng/l. The method precision and accuracy were satisfactory with recovery percentages ranging from 96 to 111% and relative standard deviations lower than 3%. The technique is also considerably cheap, fast, and easy, and, therefore, very adequate for routing monitoring. To the authors' knowledge it constitutes the first work describing a fully automated, on-line methodology for the continuous monitoring of these compounds in water.

Keywords: Endocrine disrupters; Solid-phase extraction; Steroids; Estrogens

1. Introduction

The presence of endocrine disrupting compounds (EDCs) in the environment has become a hot topic to the point that its study starts rivalling other priority health concerns such as the environmental pollution by carcinogenic compounds [1].

From the various groups of substances with reported endocrine-disrupting properties [2], the female sex hormones and the synthetic steroids are considered as the most potent estrogenic compounds. However, these groups of substances have received up till now little attention, perhaps because they have been found in the environment at very low con-

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centrations (ng/l range) [2–11]. Nowadays, the widespread use of birth control pills formulated with these potent estrogenic and progestational chemicals, capable to induce estrogenic responses in fish at concentrations as low as 1 ng/l, has alerted scientists about the potentially dangerous consequences of their presence in the aquatic environment.

The number of analytical methodologies currently available for determination of estrogens and progestogens in water is limited. These methodologies are based on either biological techniques [3–5,9,12,13] or chromatographic techniques [6–8,10,11,14].

In this study, a fully automated on-line procedure, based on solid-phase extraction–liquid chromatography–diode array detection (SPE–LC–DAD), for determination of female sex hormones and related synthetic compounds in water samples of different origin is presented. The procedure overcomes certain disadvantages of the biological techniques (limited availability of specific antisera, cross-reactivities) and of off-line chromatographic procedures (complicated, time-consuming extraction and purification steps), and represents, to the authors' knowledge, the first work describing the application of a fully automated on-line methodology to the analysis of estrogens and progestogens in water. Previously, two other attempts have been made to analyze some of these compounds in water by on-line methodologies [15,16]. However, such approaches, as described, were not amenable for automation and, in addition, had not been specifically designed for the determination of estrogens or progestogens. Thus, Ramsey et al. analyzed estrone, along with two other drugs, in water by on-line supercritical fluid extraction coupled with ultraviolet–visible diode array liquid chromatography–mass spectrometry; whereas Jahr determined ethynyl estradiol by a procedure developed for the analysis of phenols in water, based on sample-acetylation and automatic on-line SPE–gas chromatography–mass spectrometry.

2. Experimental

2.1. Chemicals

The compounds studied were the natural estrogens estradiol, estriol, and estrone; the synthetic estrogens ethynyl estradiol, mestranol, and diethylstilbestrol; the naturally occurring hormone progesterone, and the synthetic progestogens levonorgestrel and norethindrone.

Pure standards of both natural and synthetic estrogens and progestogens 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 mixtures of all the analytes were prepared at various concentrations by appropriate dilution of the stock solutions in methanol and subsequent spiking of LC-grade water. The final standard 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. Equipment

Preconcentration of the samples was performed on an automated sample preparation system (Prospekt, Spark Holland, The Netherlands) which consists of a cartridge exchange module, a solvent delivery unit (SDU) and a low-pressure six-port valve connected on-line to the chromatographic system. 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, Deventer, The Netherlands), and the polymeric cartridges PLRP-S (15–25 μm) (Polymer Laboratories, Church Stretton), HySphere-Resin-GP (5–15 μm) (Spark Holland, The Netherlands), and Oasis HLB (30–60 μm) (Waters, Milford, MA, USA).

The chromatographic system consisted of a Waters 600-MS solvent delivery unit with a 20-μl-injection loop and a Waters 996 photodiode array detector (Waters, Millipore, MA, USA). 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).

2.3. On-line trace enrichment

In the optimized method, fully automated on-line trace enrichment of the samples was performed by passing 200 ml of the water sample at 4 ml/min

through a PLRP-S cartridge (Polymer Laboratories, Church Stretton) 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 2 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. All steps of the sample preparation were programmed on, and automatically controlled by the Prospekt software.

2.4. Chromatographic conditions

The chromatographic conditions used in this study had been previously optimized for the analysis off-line of the same group of compounds in water [14]. These conditions were only slightly modified in order to simultaneously perform the loading of the sample onto the cartridge and regeneration of the chromatographic column with 100% acetonitrile followed by re-equilibration with the initial mobile phase conditions. Elution of the retained analytes from the cartridge onto the analytical column was performed, as in the off-line method, with a gradient elution from 10% acetonitrile in water to 100% acetonitrile in 40 min at 1 ml/min.

UV detection was performed at 200, 225, and 240 nm. Two hundred nanometers was used for quantitation of the estrogens and 240 nm for quantitation of the progestogens. Two hundred and twenty five nanometers, the wavelength at which all analytes exhibit some absorption, was recorded to aid identification through ratioing between the peak intensities recorded at the various wavelengths. UV spectra from 190 to 600 nm were also recorded with the same aim of aiding identification through the comparison with libraries and for peak purity assessment.

2.5. Environmental samples

Various types of environmental water samples, including drinking water, groundwater, surface water, and a sewage treatment plant (STP) effluent, were collected in precleaned amber glass bottles and kept at 4°C in the dark until analysis. Samples with high organic matter content, i.e. the surface water and the STP effluent, were filtered through a glass fiber filter (0.45 μm pore size) prior to analysis in order to remove suspended particles and avoid subsequent clogging of the SPE cartridge.

3. Results and discussion

3.1. On-line trace enrichment

In order to assess the best conditions for the on-line extraction and analysis of the tested compounds in aqueous samples, several preliminary experiments were run.

For selection of both the sample volume and the SPE sorbent, various sample volumes of LC-grade water spiked with the mixture of the test compounds were analyzed using all four SPE cartridges specified in the Experimental section. Table 1 shows the recovery percentages obtained in this study. The recoveries were calculated from the peak areas obtained for each analyte in the analysis of the spiked water samples as percentages of the peak areas obtained from the direct chromatographic injection (20 μl) of equivalent amounts of the standard mixtures in methanol.

As it can be seen, all three polymeric cartridges showed similar, good extraction capacity and elution efficiency towards all the analytes regardless of the sample volume used. In the case of the C_{18} cartridge, on the contrary, the estriol experimented breakthrough, as a consequence of its capacity factor, even with the smallest of the sample volumes tested (50 ml), and consequently this sorbent received no further consideration.

The recovery percentage obtained for diethylstilbestrol was with all the cartridges tested somewhat lower than that obtained for the other analytes studied. Since a too fast loading flow-rate of the sample could be responsible for such low recovery, due to non-equilibrium processes, 200 ml aliquots of spiked LC-grade water were analyzed again with the three polymeric sorbents but using in this case a loading flow-rate of 2 ml/min, instead of the 4 ml/min employed in the previous experiment. However, no dependence of the extraction efficiency with respect to the loading flow-rate was observed (data not reported), neither for diethylstilbestrol nor for the other analytes, and the low recovery obtained for

Table 1
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 (sample loading flow-rate=4 ml/min)

SPE cartridge	RP-18	OASIS	HySphere	PLRP-S	RP-18	OASIS	HySphere	PLRP-S	OASIS	HySphere	PLRP-S
Sample volume (ml)	50				100				200		
Spiking level (μg/l)	10				10				1		
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
Mestranol	88	90	86	88	94	93	88	89	104	99	103
Average	91	92	88	91	94	96	92	94	97	93	94

diethylstilbestrol was attributed to a phenomenon previously observed by the authors [14], according with which some kind of equilibrium process between two different isomeric forms of the compound would take place.

According with the results obtained in the previous experiments, a loading flow-rate of 4 ml/min and a sample volume of 200 ml were estimated as optima. Loading flow-rates greater than 4 ml/min and sample volumes larger than 200 ml, which would have obviously improved the analysis time and the sensitivity of the method, respectively, were not evaluated because preliminary test experiments with real samples under such conditions were observed to provoke clogging of the sorbent despite the previous filtration of the sample.

Finally, from the three polymeric cartridges, the PLRP-S was preferred to the others, because it gave a comparatively better recovery for diethylstilbestrol than the HySphere-Resin-GP cartridge, and because, unlike the Oasis cartridge, it did not provoke band-broadening. Fig. 1 shows typical chromatograms obtained from the analysis using the optimised extraction procedure of LC-grade water spiked with the mixture of the analytes at 1 μg/l at the three wavelengths monitored. Under the selected conditions the analysis time is 103 min.

3.2. Method performance

The method performance was evaluated through estimation of the linearity, repeatability, accuracy, and sensitivity of the method.

For quantitation the external standard method was used. The Millennium 32 software application (from Waters, Milford, MA, USA) was used to assist in the

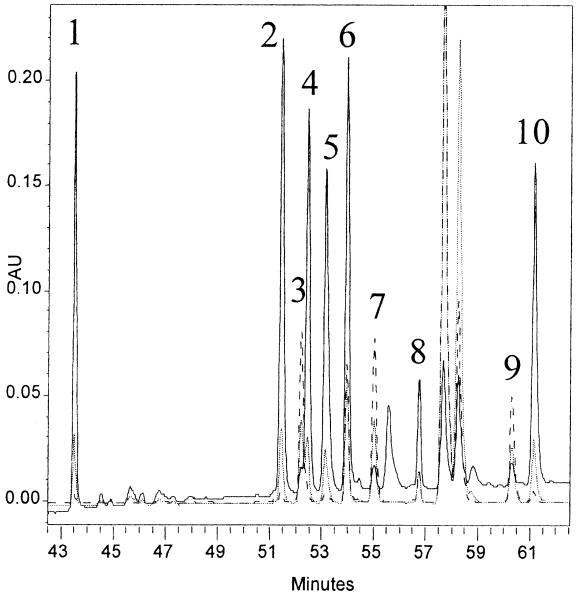

Fig. 1. Chromatograms obtained from the on-line analysis of LC-grade water spiked with a mixture of the analytes at 1 μg/l. —— 200 nm; ····· 225 nm ------ 240 nm. Peak identification: estriol (1); estradiol (2); norethindrone (3); ethynyl estradiol (4); estrone (5) diethylstilbestrol (6); levonorgestrel (7); diethylstilbestrol isomer? (8); progesterone (9); mestranol (10).

quantitation, based on peak areas, of standards and samples.

Five-point calibration curves were constructed, using a least-square linear regression analysis, from the application of the overall method to 200 ml aliquots of LC-grade water spiked with the analytes at concentrations ranging from 25 ng/l to 10 μg/l. The calibration curves were linear with correlation coefficients (r^2) higher than 0.994 for all compounds (Table 2).

The overall method repeatability and accuracy were determined from the analysis of six replicates of LC-grade water (200 ml) spiked with a standard mixture of the analytes at 10 μg/l (see Table 2). Both the repeatability, with relative standard deviations (RSD) ranging from 0.9 to 3.4%, and the accuracy, with recovery percentages (%*R*) ranging between 96 and 112 for all compounds, were satisfactory and indicated good performance of the method developed. One advantage of automated on-line methodologies, as compared with off-line methodologies, is that more reproducible results are obtained, provided that the manipulation of the samples is reduced to a minimum or completely avoided. Likewise, elimination of common intermediate steps of off-line methodologies as, for instance, the drying step carried out to reduce the volume of the extract that often leads to loss of the more volatile compounds, makes this type of approach more accurate.

The sensitivity is another parameter usually enhanced in on-line systems since the whole sample, instead of an aliquot of the final extract as in off-line systems, is transferred to the chromatographic system. Thus, the sensitivity reached with on-line systems can be about one order of magnitude better than that obtained with similar off-line approaches.

The instrumental detection limits calculated for each analyte using a signal-to-noise ratio of 3 from the analysis of spiked LC-grade water are listed in Table 2. These detection limits can be extrapolated to the analysis of fairly clean waters, such as drinking water, ground water or surface water. However, in the case of more complex samples, such as sewage treatment plant influents and effluents, the method sensitivity is seriously compromised by the matrix effect.

Fig. 2 shows the chromatograms obtained, at the wavelength characteristic for the group of estrogens (200 nm), from the analysis of a drinking water sample as it is and of the same sample spiked with a mixture of the analytes at 100 ng/l. A similar example for a groundwater sample, but in this case corresponding to the wavelength characteristic for the group of progestogens (240 nm), is shown in Fig. 3.

Both types of samples give pretty similar chromatograms at the wavelengths monitored and as it can be seen in the representative figures both the estrogens and the progestogens can be analyzed with the present methodology in both drinking water and groundwater at concentrations distinctly below 100 ng/l, which is also the case of relatively unpolluted surface waters.

In the case of highly polluted surface water (Fig. 4) and STP effluents (Fig. 5), however, the accurate

Table 2
Analytical data corresponding to the analysis of estrogens and progestogens in water[a]

Compound	t_R (min)	R^2	Detection limits (ng/l)	RSD	Recovery (%)
1. Estriol	15.50	0.9969	15	3	103
2. Estradiol	23.44	0.9962	15	3	104
3. Norethindrone	24.21	0.9981	15	3	105
4. Ethynyl estradiol	24.44	0.9969	15	3	111
4. Estrone	25.15	0.9962	10	2	112
5. Diethylstilbestrol	25.97	0.9943	15	3	96
6. Levonorgestrel	27.02	0.9988	15	1	101
7. Progesterone	32.31	0.9995	20	1	102
8. Mestranol	33.15	0.9967	20	1	111

[a] Calibration range, 25 ng/l–10 μg/l (five data points); detection limits at $S/N=3$.

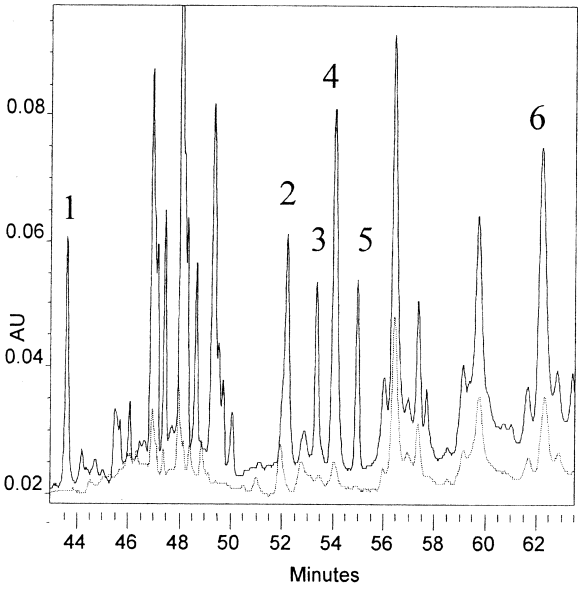

Fig. 2. Chromatograms obtained from the on-line analysis of 200 ml of drinking water (------) and of drinking water spiked with a standard mixture of the analytes at 100 ng/l (——). $\lambda = 200$ nm. Peak identification: estriol (1); estradiol (2); ethynyl estradiol (3); estrone (4); diethylstilbestrol (5); mestranol (6).

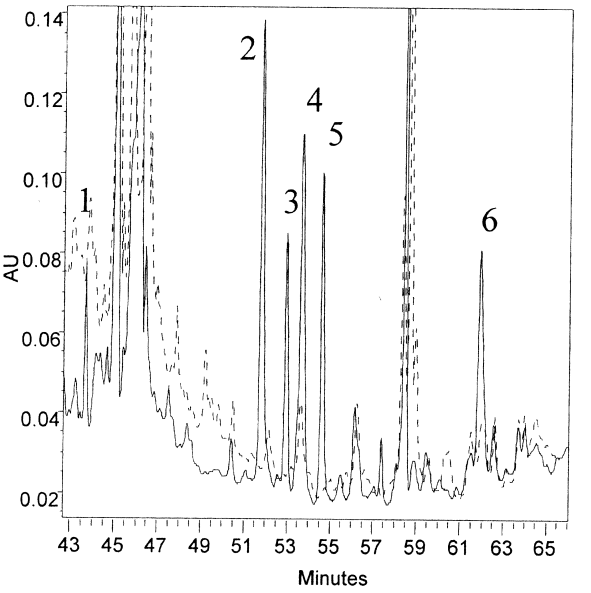

Fig. 4. Chromatograms obtained from the on-line analysis of 200 ml of highly polluted river water (------) and highly polluted river water spiked with a mixture of the analytes at 500 ng/l (——). $\lambda = 200$ nm. Peak identification: estriol (1); estradiol (2); ethynyl estradiol (3); estrone (4); diethylstilbestrol (5); mestranol (6).

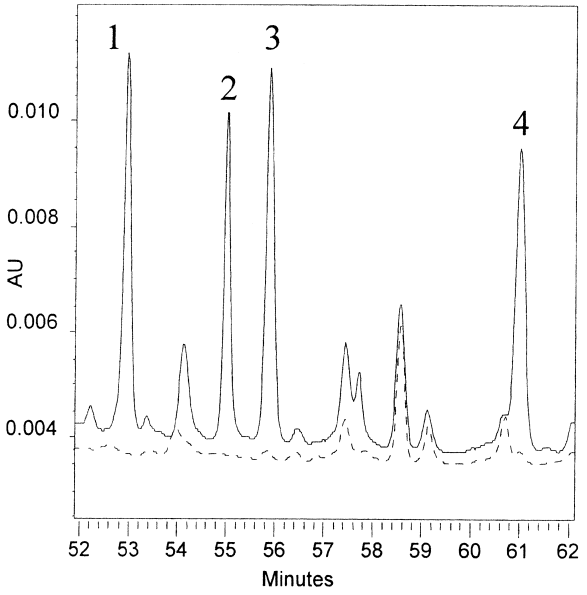

Fig. 3. Chromatograms obtained from the on-line analysis of 200 ml of groundwater (------) and of groundwater spiked with a standard mixture of the analytes at 100 ng/l (——). $\lambda = 240$ nm. Peak identification: norethindrone (1); diethylstilbestrol (2); levonorgestrel (3); progesterone (4).

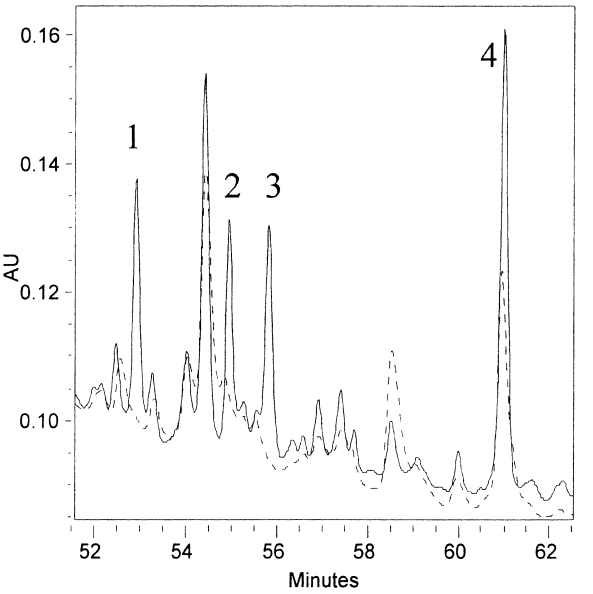

Fig. 5. Chromatograms obtained from the on-line analysis of a STP effluent (------) and of a STP effluent spiked with a standard mixture of the analytes at 500 ng/l (——). $\lambda = 240$ nm. Peak identification: norethindrone (1); diethylstilbestrol (2); levonorgestrel (3); progesterone (4).

quantification of both the estrogens and the progestogens can only be performed at concentrations of the compounds greater than 200–300 ng/l, depending on the complexity of the particular sample.

Therefore, the methodology here described allows for the precise and accurate determination of the most relevant estrogens and progestogens in various environmental waters at concentrations ranging between 25–300 ng/l and 10 μg/l. According with the available toxicological data [7,12,17–23], the most potent estrogenic compounds estradiol and ethynyl estradiol are capable to induce alterations in the endocrine system of life organisms at concentrations in water as low as 1 ng/l [22,23] whereas the rest of the compounds studied in this work have estrogenic potencies one to several orders of magnitude lower than those of estradiol and ethynyl estradiol [18–20]. In consequence, the sensitivity achieved with the present methodology would permit to alert of the presence of most of the target compounds at potentially harmful concentrations in most environmental waters. However, in the case of the most potent compounds estradiol and ethynyl estradiol a more sensitive and selective detector, such as a mass spectrometer, or a more selective extraction/purification procedure, based perhaps on the use of immunosorbents (unfortunately not yet available for these particular analytes), would be necessary to permit a similar warning about their eventually dangerous presence in the aquatic environment.

Advantages of the methodology in its present form are automation, speed, and cost. Thus, up to 16 samples can be analyzed in a fully automated, unattended way. The no requirement for sample manipulation, other than the filtration step carried out only in the case of samples with high turbidity, results in the already discussed improved repeatability and accuracy, and in reduced analysis time (103 min per sample). On the other hand, the simplicity of the technique obviates the need for highly qualified or experienced staff, which along with an estimated global price of the sample preparation instrumentation of about US$ 30 000, hardly any maintenance expenses, and operating costs reduced to the LC mobile phase, the cartridges, and a low consumption of compressed air, makes the technique also considerably cheap.

It is important to point out that for this particular analysis the cartridge, contrary to the manufacturers' general specifications, can not be reused. Fig. 6 shows the peak areas obtained for estriol and mestranol, the analytes with the shortest and the longest retention times, respectively, from the replicate analysis ($n=18$) of 200 ml spiked groundwater using the same cartridge. As it can be seen, based on the peak areas obtained, the cartridge could be reused up to 12 times in the analysis of the most apolar compounds, such as mestranol. However, in the case of more polar compounds, such as estriol, only a single use is recommended because the retention capacity exhibited by the cartridge decreases with every additional use. Nevertheless, some peak tailing was also observed to appear from the seventh analysis on for all compounds, and so, the number of times for the cartridges to be reused should not be larger than six in any case.

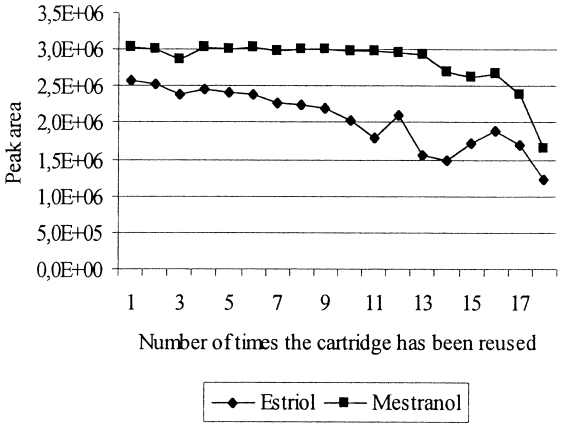

Fig. 6. Peak areas obtained for estriol and mestranol from the replicate analysis ($n=18$) using the same cartridge of spiked (10 μg/l) groundwater.

4. Conclusions

The information gathered from the various monitoring programs carried out up till now to assess the impact of the human and environmental exposure to EDCs has alarmed scientists and environmental and health institutions about the potentially dangerous consequences of such exposure and evidenced the need for further, extensive research in this area.

In this context, a methodology for the determi-

nation in water of the most relevant estrogens and progestogens, in terms of estrogenic potency and environmental occurrence, was developed. The on-line SPE–LC–DAD approach here presented allows for the simultaneous analysis of the target compounds in various types of environmental water, including drinking water, groundwater, surface water and STP effluents at the ng/l level and in up to 16 samples, the maximum number that the Prospekt can process, in a fully automated, unattended way. It presents a considerable number of advantages as compared to the previously published off-line methodologies. Thus, the approach is highly precise, accurate, cheap, and fast, and, therefore, very adequate for the routine monitoring, required for both assessment of the current environmental occurrence of these priority substances and for future water quality control.

To the authors' knowledge, it constitutes the first description of the application of a fully automated on-line methodology to the quantitation of these compounds in water, and based on the present results further hyphenation of the described system with a mass spectrometer and/or, eventually, with a previous isolation step based on receptor affinity chromatography or on immunsorbents, should greatly enhance the applicability of the method by improving the sensitivity and selectivity.

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