Multivariate calibration-assisted high-performance liquid chromatography with dual UV and fluorimetric detection for the analysis of natural and synthetic sex hormones in environmental waters and sediments

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A B S T R A C T

A green method is reported based on non-sophisticated instrumental for the quantification of seven natural and synthetic estrogens, three progestagens and one androgen in the presence of real interferences. The method takes advantage of: (1) chromatography, allowing total or partial resolution of a large number of compounds, (2) dual detection, permitting selection of the most appropriate signal for each analyte and, (3) second-order calibration, enabling mathematical resolution of incompletely resolved chromatographic bands and analyte determination in the presence of interferents. Consumption of organic solvents for cleaning, extraction and separation are markedly decreased because of the coupling with MCR-ALS (multivariate curve resolution/alternating least-squares) which allows the successful resolution in the presence of other co-eluting matrix constituents. Rigorous IUPAC detection limits were obtained: $6-24$ ng L^{-1} in water, and $0.1-0.9$ ng g^{-1} in sediments. Relative prediction errors were $2-10\%$ (water) and $1-8\%$ (sediments).

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1. Introduction

Natural and synthetic hormones (estrogens, progestagens and androgens), phytoestrogens and some industrial chemical compounds constitute a group of contaminants called endocrine disruptors (EDs) (Gutendorf and Westendorf, 2001). The presence of EDs in the environment represents a specific pollution threat with potential ecological and human health implications (Gutendorf and Westendorf, 2001; Solomon and Schettler, 2000).

Estrogens and progestagens are constantly excreted by humans, reaching the aquatic environment through sewage systems and, therefore, domestic wastewaters are established as a main source of contamination for these EDs (Gabet et al., 2007; Besse and Garric, 2009). Sources of androgens include, in addition to treated domestic wastewater, livestock breeding, pulp mills and degradation of natural phytosterols (Streck, 2009; Liu et al., 2012).

The determination of sexual hormones in aquatic bodies and related environmental samples such as sediments is a very important activity in modern steroid hormone analysis (Görög, 2011). While numerous reports have been published on the determination of estrogens in environmental waters and, to a lesser extent, sediments, studies on progestagenic and androgenic hormones are scarce (Besse and Garric, 2009; Streck, 2009). Several comprehensive reviews about this subject have been published (Gabet et al., 2007; Streck, 2009; Görög, 2011; Kuster et al., 2004). Gas chromatography-mass spectrometry (GC—MS), which usually requires a derivatization step, has been progressively replaced by liquid chromatography (LC)-based techniques coupled with MS or tandem MS for quantification of estrogenic, progestagenic and androgenic compounds in complex environmental matrices. The latter techniques offer outstanding sensitivity and selectivity, although they employ sophisticated detectors and strict extraction and clean up processes are mandatory before their application (Streck, 2009).

A current trend in environmental analysis is to avoid sample pre-processing steps and long chromatographic runs, exploiting the ability of modern data processing tools for mathematical resolution of coeluting components. Needless to say, analytical methods for pollutants quantification should not contribute with

additional contamination. Within the past few years, a new set of methods has arisen, the so-called "green analytical chemistry" (GAC) methods. The driving force has been the need to protect the environment, without negative impact on basic analytical properties (Armenta et al., 2008; de la Guardia, 2010).

The main objective of the present work was the development of a GAC method for the analysis of a significant number of sex hormones at part per trillion concentrations in surface and underground waters and sediments. The natural estrogens estriol (E3), 17β-estradiol (E2) and estrone (E1) and the synthetic 17α-ethinylestradiol (EE2) have been previously studied coupling LC-diode-array detection (DAD) data to chemometric analysis (Pérez and Escandar, 2014). In the present work, single-run dual DAD and fluorescence detection (FLD) are applied for the determination of eleven analytes involving natural (E3, E2, E1) and synthetic [EE2, diethylstilbestrol (DES), hexestrol (HEX), mestranol (MEST)] estrogens, endogenous [progesterone (PROG)] and synthetic [norethisterone (NOR), levonorgestrel (LEV)] progestagens, and a common precursor of male and female sex hormones, androstenedione (AE) (Fig. 1). The dual detection allows us to quantify: (1) estrogens, through the intense fluorescence displayed by most of them in the employed mobile phase, and (2) the remaining non-fluorescent hormones by their UV absorption properties. The benefits obtained by combining the applied analytical method with the chemometric algorithm multivariate curve resolution with alternating least-squares (MCR-ALS) (Tauler et al., 2009) are demonstrated. Although the combination of LC and second-order calibration has been reported in the literature (Escandar et al., 2014), the limits of the technique are still unknown in terms of the number of analytes that can be quantified in highly interfering media. To the best of our knowledge, this is the first time that eleven sex hormones are evaluated in challenging media using a GAC method, and second-order calibration is applied to both high-performance liquid chromatography (HPLC)-DAD and HPLC-FLD matrices measured for a single chromatographic run.

2. Materials and methods

2.1. Reagents and solutions

AE, DES, E2, EE2, E3, E1, HEX, LEV, MEST, NOR, and PROG were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Water was purified using a MilliQ system (Millipore, Bedford, USA). Solvents were filtered through 0.22 μm nylon filters.

Stock solutions of all analytes of about 2000 μg mL^{-1} were prepared in methanol. From these solutions, more diluted methanol solutions (around 100 μg mL^{-1}) were obtained. Working solutions were prepared immediately before their use by taking appropriate aliquots of diluted methanol solutions, drying the solvent under a nitrogen stream and adding acetonitrile and water (50:50 v/v) to the desired concentrations.

2.2. Apparatus

Chromatographic measurements were carried out on an HP 1200 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with degasser, quaternary pump, a manual injector fitted with a 20 μL loop, a DAD, an FLD, and the HP ChemStation software package for instrument control, data acquisition and data analysis. HPLC separations were performed on a Poroshell 120 EC (4.6 × 100 mm, 2.7 μm particle size) column (Agilent Technologies, Santa Clara, CS, USA).

2.3. HPLC procedure

Data matrices were collected every 1.8 s using wavelengths from 200 to 330 nm in steps of 1 nm for the DAD, and every 1.5 s from 295 to 350 nm in steps of 1 nm for the FLD, setting the excitation wavelength at 275 nm and the slit widths at 1 nm. HPLC-DAD matrices of size 580 × 131 and HPLC-FLD matrices of size 162 × 56 (time and spectral data points respectively) were saved in ASCII format, and transferred to a PC for subsequent manipulation. The mobile phase used was a 50:50 (v/v) mixture of water and acetonitrile, delivered at a flow rate of 1.0 mL min^{-1} with a chromatographic system operating under isocratic mode.

2.4. Calibration and validation samples

A calibration set of ten samples containing E3, E2, EE2, HEX and MEST in the range 0–50 ng mL^{-1} and the remaining compounds in the range 0–100 ng mL^{-1} was prepared (Table S1 of Supplementary data). These concentrations were selected considering the low
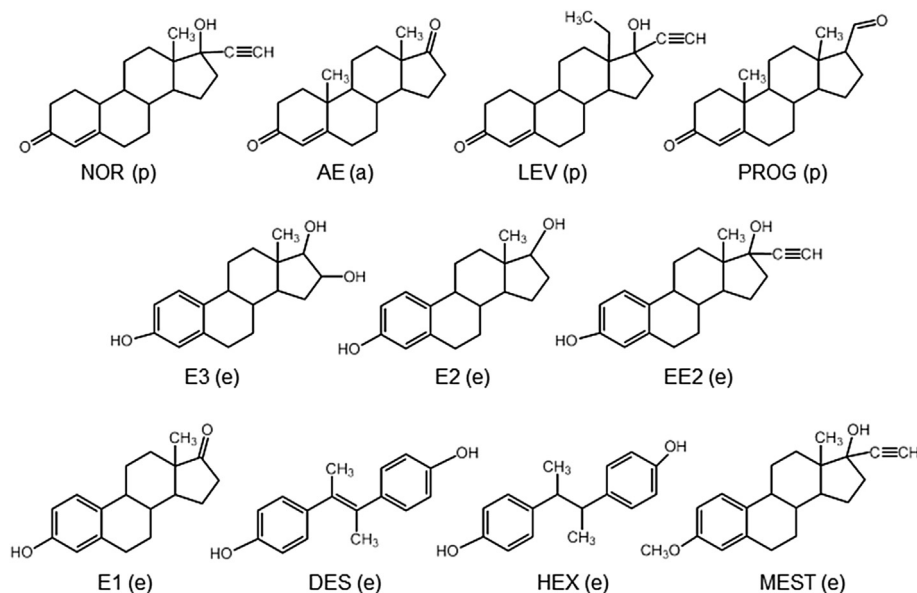

Fig. 1. Structures of the evaluated estrogens (e), progestagens (p) and androgens (a).

levels of sex hormones usually found in natural samples (see below) and no efforts were made to establish the upper concentration of the linear range. Eight samples of the set corresponded to the concentrations provided by a semi-factorial design for four overlapped analytes (E1, DES, AE and HEX) and equally spaced concentrations for those analytes with resolved bands. The remaining calibration samples were a blank solution (with no addition of any of the eleven analytes) and a mixture of all studied analytes at intermediate concentrations (e.g. ~ 25 and 50 ng mL^{-1}). A validation set of ten samples was additionally prepared, containing the analytes in different concentrations than those used for calibration. Specific concentrations were taken as random numbers generated within the calibration domain.

2.5. Water samples

Three different water samples (mineral, underground and river) were analyzed. Underground (Funes City) and river water (Paraná River) samples were collected in amber glass bottles, previously cleaned with methanol and Milli-Q water, and stored at 4 °C after sampling. Mineral water (Mendoza) was evaluated as purchased, while underground and river samples were filtered with filter paper before their use.

Because none of the real samples contained the investigated compounds at larger levels than the attained detection limits, a recovery study was performed spiking all water samples with standard solutions of the analytes. For estrogens (except E1 and DES), the ranges were 10–20 ng L^{-1} (low), 25–35 ng L^{-1} (medium) and 40–52 ng L^{-1} (high), whereas for the remaining analytes they were 19–32 ng L^{-1} (low), 46–65 ng L^{-1} (medium) and 81–99 ng L^{-1} (high). The solid-phase extraction (SPE) procedure was carried out using SPE disks Empore Octadecyl C18 (Supelco, Bellefonte, PA, USA). The membrane was conditioned with 1 mL of methanol and then the extraction of 100 mL of the sample was carried out in approximately 10 min per sample. This flow rate is in the optimum range for maximum breakthrough volume (Hagestuen et al., 2000). The retained compounds were eluted with 0.5 mL methanol, and this solvent was evaporated under a nitrogen stream. Then, the residue was reconstituted with 0.200 mL of mobile phase, filtered by a nylon filter before injection and finally subjected to the same chromatographic analysis as the calibration samples. The preconcentration factor was 1:500.

2.6. Sediment samples

Sediment samples from a water treatment plant (Rosario, Argentina), Paraná river and Carcarañá river were collected in glass bottles, previously cleaned with methanol and Milli-Q water. Since these samples did not contain detectable levels of the evaluated compounds, they were spiked with standard methanol solutions in order to obtain concentration levels the range 2.5–24.3 ng g^{-1}. The fortified samples were then frozen and lyophilized in a Liotop L101 Liobras dryer (San Carlos, Brazil). Finally, they were ground using a mortar and stored at −15 °C until analysis. For the extraction procedure, 2.00 g of lyophilized sediment were placed into a 25 mL beaker and treated with 5 mL of methanol. The mixture was ultrasonic extracted for thirty minutes at room temperature and then was centrifuged at 10,000 g for ten minutes. A portion of the supernatant was placed in a 100 mL volumetric flask, dried under a gentle nitrogen stream and reconstituted with water to the mark. The resulting solution was subjected to the same SPE procedure used for the water samples with a preconcentration factor of 1:500.

2.7. MCR-ALS algorithm and software

The MCR-ALS theory is well documented in the literature (Tauler et al., 2009) and only a brief description is included in the Supporting Information. The data were handled using the MATLAB computer environment (MATLAB Version, 2011b). The calculations involving MCR-ALS were performed using MVC2, a new version of the already reported MATLAB graphical interface toolbox (Olivieri et al., 2009), freely available on the Internet (www.iquir-conicet.gov.ar/descargas/mvc2.rar).

3. Results and discussion

3.1. Preliminary considerations

In accordance to the premise of developing a greener chromatographic method, the working conditions here employed were selected considering that reliable results should be obtained employing a mobile phase with a low amount of organic solvent and in the shortest possible overall chromatographic time.

Fig. 2 shows typical DAD and FLD chromatograms at selected wavelengths for absorbance ($\lambda = 240$ nm) and excitation/emission (λex $= 275$ nm, λem $= 310$ nm) in a case of a calibration sample under our working conditions, and the corresponding contour plots of data matrices used for subsequent processing.

All studied analytes present absorption in the UV region (Fig. 3A); therefore, they can be chromatographically measured with a DAD at sub-part per billion after suitable pre-concentration. Most of the studied estrogens were also highly fluorescent in the used mobile phase (Fig. 3B), and this fact was exploited for their determination at even lower concentrations than UV/DAD. Specifically, while low or non-fluorescent compounds were chromatographically quantified through their UV signals (namely, NOR, DES, AE, LEV, PROG and E1), the estrogens E3, E2 and EE2 were determined by fluorescence. On the other hand, the synthetic estrogens HEX and MEST, which display both intense absorbance and fluorescence signals were, in principle, determined using both types of detectors.

The resolution for some chromatographic bands of the DAD system is only partial (Fig. 2). The picture is even more critical when the test sample is no longer a synthetic one prepared in mobile phase, but a real sample, usually consisting of a significantly more complex matrix. This latter situation affects both the DAD and FLD systems through severe band overlapping. Therefore, the use of multivariate calibration through the processing of HPLC-spectral second-order data is entirely justified.

Prior to constructing the experimental matrices, the characteristics of this type of data must be considered. In chromatographic analysis, it is very common to observe the lack of repeatability in the retention time and band shape of an analyte between successive runs. As a result, the three-dimensional array formed with the chromatographic-spectral matrices obtained loses the property of trilinearity (Olivieri and Escandar, 2014). Although this fact represents a serious obstacle for algorithms which demand the trilinearity of the data (Olivieri and Escandar, 2014), algorithms such as MCR-ALS do not require this condition. They represent a valuable tool for the processing of this type of data, for example by performing matrix augmentation in the temporal direction (Tauler et al., 2009). However, in the system under study, an additional problem must be taken into account: some analytes exhibit very similar absorbance and fluorescence spectra (Fig. 3). In this situation, if the full DAD and FLD chromatograms are processed, unsuitable results are obtained because the mathematical pseudorank is smaller than the chemical rank (Olivieri and Escandar, 2014). To overcome this inconvenience, MCR-ALS was applied with matrix
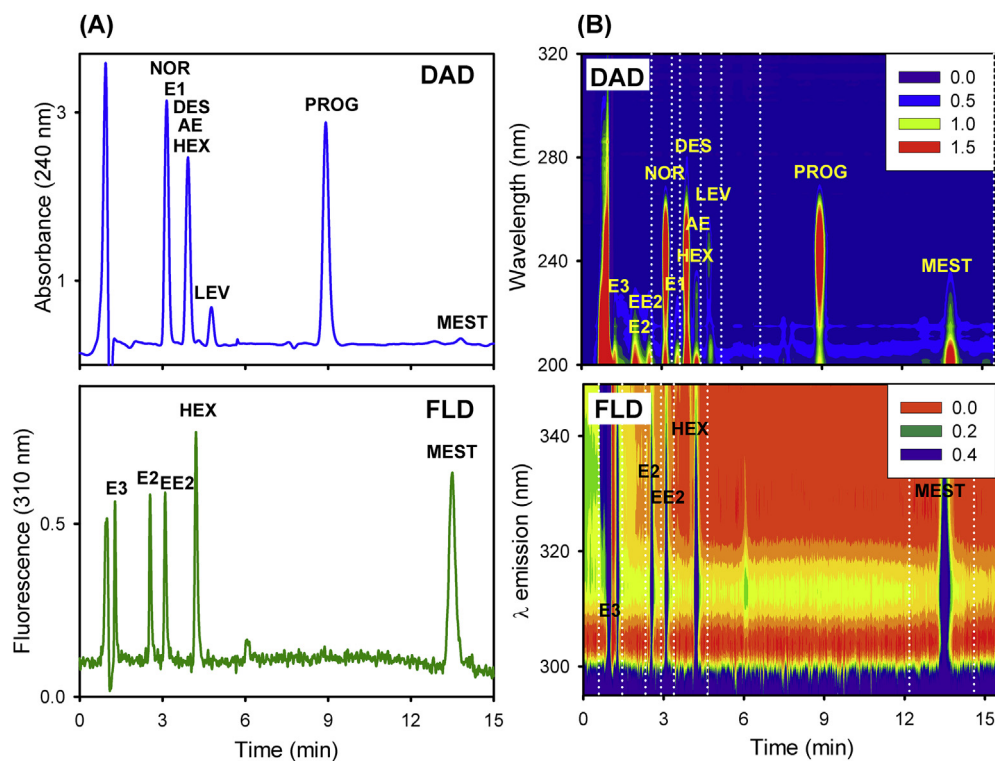
Fig. 2. DAD (blue) and FLD (green) chromatograms of a selected calibration sample (sample 10, see Table S1 of Supporting Information) (A), and the corresponding two-dimensional contour plots (B). The excitation wavelength for the FLD detection was 275 nm. In (B) the color bars indicate the vertical scales (mAU and UF for DAD and FLD, respectively) and the dotted white lines delimit the selected chromatographic/spectral regions used for data processing as indicated in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

augmentation in the temporal direction in various selected time ranges, ensuring that each partial chromatographic region includes analytes with different spectral profiles (Table 1).

3.2. Analysis of calibration and validation sets

MCR-ALS data processing comprised the building of augmented matrices in the elution time direction containing, for each time region, a validation sample data and the calibration data matrices. The number of components in each augmented matrix was estimated by principal component analysis, and justified taking into account the presence of the corresponding analytes and background signals. Non-negativity restrictions were applied in both modes; unimodality restriction was applied in the elution time mode to the signals corresponding to the analytes. The selected ALS convergence criterion was 0.01% (relative change in fit for successive iterations), and in validation samples convergence was achieved in less than 20 iterations. The residual fits for the DAD were lower than 0.04 mAU (milli absorbance units), while those corresponding to FLD were about 0.01 UF (arbitrary units of fluorescence), which is ca. 1% with respect to the maximum intensity measured. After convergence of the ALS optimization for each sample, the constituents were identified and quantification was carried out with the aid of the corresponding pseudo-univariate calibration curves. Table 2 shows the parameters obtained for the latter regression curves corresponding to a typical validation sample. The concentration prediction of each analyte proceeded by interpolation into the corresponding pseudo-univariate score-concentration calibration plot.

Fig. 4 displays the good recovery results in validation samples in addition to the elliptical joint confidence region (EJCR) (González et al., 1999) test for the slope and intercept of the plot

corresponding to each analyte. Because all ellipses include the theoretically expected values of (1,0) for slope and intercept, respectively, the accuracy of the applied methodology for these compounds in validation samples can be claimed. The statistical results corresponding to validation samples are completed with the parameters shown in Table S2 of the Supplementary data.

Although HEX and MEST are successfully determined with both types of detections (Fig. 4), the sizes of their ellipses resulting from the predicted concentrations using DAD are significantly larger than those corresponding to FLD, suggesting a better precision when the latter detector is employed. Therefore, the quantification of HEX and MEST in real samples was only carried out through HPLC-FLD data.

3.3. Analysis of real samples

The resolution of the samples selected as examples of environmental matrices for evaluating the proposed methodology represents a real analytical challenge (Fig. 5). However, MCR-ALS achieves the so-called "second-order advantage", which avoids the major obstacle of traditional zeroth-order calibration methods applied to complex mixtures: the requirement of interference removal before the quantitative analytical method is applied (Olivieri, 2008).

MCR-ALS data processing was similar to that for validation samples, but in addition to non-negativity in both modes and unimodality in the time mode restrictions, the correspondence restriction was applied to most samples, which fixes the sequence and the presence or absence of components in specific matrices (Tauler et al., 2009). In real samples, with an unknown number of constituents, the number of components was estimated as in validation (see above) and varied between 6 and 8, depending on the
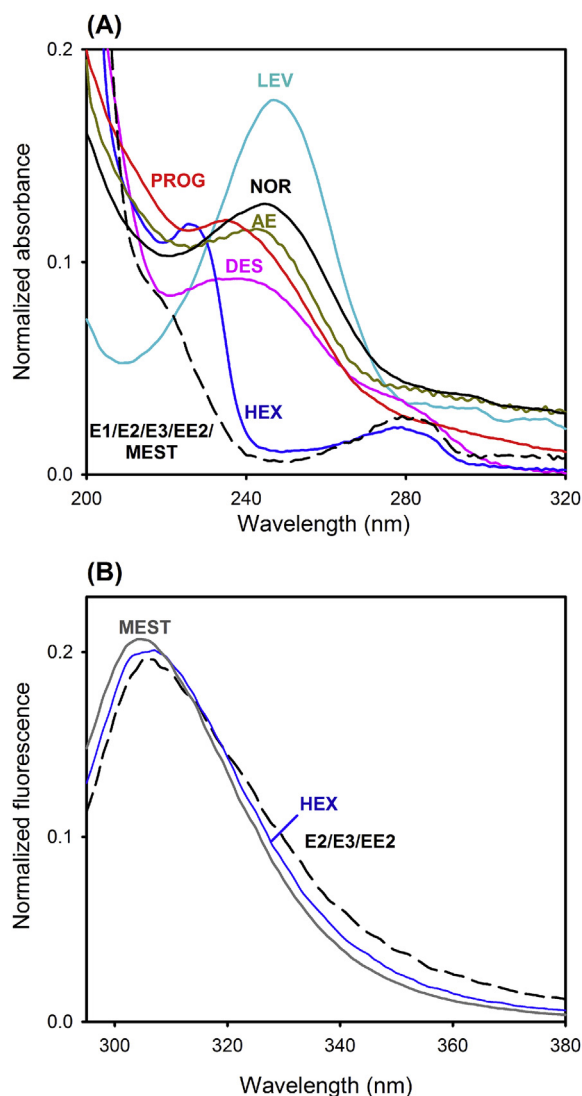
Fig. 3. Normalized absorption (A) and fluorescence emission (B) spectra for the assayed endocrine disruptors in acetonitrile-water (50:50, v/v). (A) NOR (black), DES (pink), AE (dark yellow), HEX (blue), LEV (cyan), PROG (red) and E1, E2, E3, EE2 and MEST (dashed-black). (B) MEST (gray), HEX (blue) and E2, E3 and EE2 (dashed-black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Selected chromatographic/spectral ranges used for MCR-ALS data processing.

Analyte	Time (min)	Wavelength (nm)
DAD		
NOR	2.80−3.30	200−330
E1	3.30−3.60	200−330
DES/AE/HEX	3.60−4.40	200−330
LEV	4.40−5.00	215−310
PROG/MEST	6.70−15.5	200−330
FLD		
E3	0.70−1.50	290−350
E2	2.30−2.90	290−350
EE2	2.90−3.30	290−350
HEX	3.30−4.80	290−350
MEST	12.1−14.7	290−350

sample and analyzed time region.

The number of ALS iterations in these complex samples was less than 30 in most cases, with residual fits in the order of the expected

instrumental noise associated with each detector. As in validation samples, after convergence was achieved, quantification was carried out with the aid of the corresponding pseudo-univariate calibration curves.

3.4. Water samples

Concentrations of estrogens and progestagens in surface and wastewaters are normally are lower than 20 ng L^{-1} (Besse and Garric, 2009; Streck, 2009; Vulliet et al., 2008; Fernandez et al., 2007). However, larger amounts (e.g. E1, 51−3240 ng L^{-1}; E2, 451 ng L^{-1}; EE2, 178−410 ng L^{-1}; DES, 122 ng L^{-1}; NOR, 26−224 ng L^{-1}; AE, 10,500 ng L^{-1}; PROG, 3470 ng L^{-1}) (Liu et al., 2012; Vulliet et al., 2008; Aerni et al., 2004) can be sporadically found. Androgenic substances such as AE are sometimes identified in rivers associated with paper mill effluents, and concentrations in the range about 30−170 ng L^{-1} have been reported (Jenkins et al., 2003; Thomas et al., 2002). Water samples were spiked with all analytes, combining random values from the corresponding concentration ranges and, after a simple pre-concentration with a C18 membrane, each sample was processed as the validation ones. Concentrations at sub-part per trillion could be measured with a larger pre-concentration step (e.g. 1:2500) (Pérez and Escandar, 2014).

It is necessary to make a distinction between the presently proposed strategy, that only needs to remove suspended particles in some natural waters from more strict extraction and/or clean-up protocols usually employed in chromatographic analysis coupled to MS or tandem MS for the determination of sex hormones in natural waters (Streck, 2009; Görög, 2011; Vulliet and Cren-Olivé, 2011; Liu et al., 2014; Zhang et al., 2011; Kuster et al., 2009; Sun et al., 2009). In our case, because of the second-order advantage, soluble sample constituents injected in the chromatographic column along with the analytes do not interfere in the analysis, as is demonstrated with the successful MCR-ALS predictions (Table 3).

Fig. S1 and S2 (Supplementary data) show the profiles retrieved by MCR-ALS in both spectral (absorbance or fluorescence) and temporal modes for the studied analytes in a river water. The augmented time profiles in these figures contain successive sub-profiles for the unknown (river) and calibration samples. As can be appreciated, the presence of interferences in the unknown sample does not prevent the spectra to be correctly distinguished. On the other hand, Table 2 shows the good analytical parameters obtained from the MCR-ALS pseudo-univariate calibration curves for each analyte in one of the studied underwater samples selected as an example.

The obtained results for the real water samples, in terms of the EJCR test (Fig. 6), with ellipses for each type of water sample including the (1,0) expected values, indicate the accuracy of the used methodology.

Table 3 also shows the statistical results for the analyzed samples. The relative errors of prediction are very acceptable (smaller than 10%) taking into account the complexity of the studied samples. Limits of detection (LODs) were estimated based on rigorous IUPAC's recommendations, which take into account type I and II errors (false positive and false negative errors, respectively) and the error propagation from both the slope and the intercept of the pseudo-univariate MCR-ALS calibration curve (Olivieri, 2014):

$$LOD = 3.3\left(SEN^{-2}\sigma_x^2 + h_0 SEN^{-2}\sigma_x^2 + h_0\sigma_{ycal}^2\right)^{1/2} \quad (1)$$

where the factor 3.3 is the sum of t-coefficients accounting for type I and II errors at 95% confidence level, h_0 is the sample leverage at zero analyte concentration, σ_x^2 is the variance in the instrumental

Table 2
Results from the MCR-ALS pseudo-univariate calibration curves for each analyte in a typical validation sample (VS) and in a real water sample (WS) using DAD and FLD.

	Slope[a]		Intercept[a]		(r^2)[b]		$(s_{y/x})$[c]		p Value[d]	
	VS	WS[e]	VS	WS[e]	VS	WS[e]	VS	WS[e]	VS	WS[e]
DAD										
NOR	0.29(3)	0.29(2)	2.7(8)	3(1)	0.989	0.959	1.6	2.7	0.54	0.52
E1	0.23(2)	0.18(2)	−2(1)	1(1)	0.964	0.952	2.2	1.9	0.64	0.43
DES	0.48(6)	0.47(1)	−3(2)	−4(1)	0.939	0.985	8.6	1.4	0.57	0.59
AE	0.28(3)	0.28(2)	2(2)	2(1)	0.939	0.976	3.5	2.1	0.69	0.64
HEX	0.49(2)	f	−0.4(2)	f	0.992	f	1.1	f	0.81	f
LEV	0.18(3)	0.24(1)	3(1)	0.7(5)	0.863	0.989	2.9	0.9	0.68	0.52
PROG	0.37(8)	0.38 (3)	1.6(6)	1 (1)	0.998	0.986	3.5	3.8	0.62	0.68
MEST	0.62(2)	f	−0.4(3)	f	0.992	f	1.1	f	0.53	f
FLD										
E3	0.23(1)	0.29(1)	4.8(4)	−0.1(1)	0.976	0.990	0.9	0.7	0.62	0.80
E2	0.31(2)	0.41(2)	0.6(4)	−0.8(1)	0.986	0.981	0.8	1.5	0.38	0.80
EE2	0.23(1)	0.07(1)	0.4(1)	0.1(1)	0.997	0.945	0.3	0.5	0.43	0.11
HEX	0.45(1)	0.47(1)	1.1(3)	0.4(2)	0.997	0.995	0.6	0.8	0.08	0.75
MEST	0.42(4)	0.18(3)	10.6(5)	−2.0(7)	0.984	0.918	1.1	1.7	0.45	0.48

[a] Standard deviation in the last significant figure is given between parentheses.
[b] Squared correlation coefficient.
[c] Standard deviation of regression residuals.
[d] Probability associated to the IUPAC recommended F test for linearity (p > 0.05 implies linearity at 95% confidence level).
[e] The selected sample corresponds to one of the studied underwater samples.
[f] HEX and MEST in real samples were only determined by FLD.
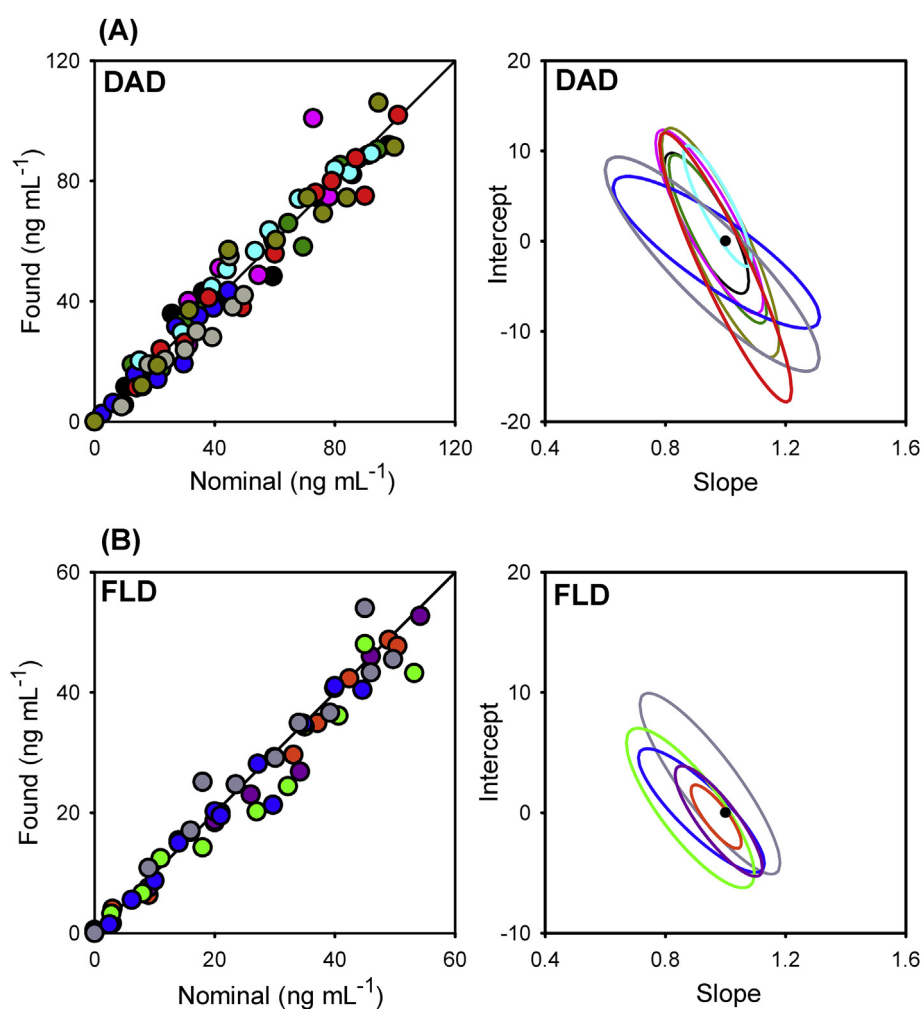
Fig. 4. Plots for MCR-ALS predicted concentrations as a function of the nominal values for NOR (black), E1 (green), DES (pink), AE (dark yellow), HEX (blue), LEV (cyan), PROG (red), and MEST (gray) using DAD (A), and for E3 (orange), E2 (violet), EE2 (light green), HEX (blue), and MEST (gray) using FLD (B) in validation samples. The right panels show the corresponding elliptical joint regions (at 95% confidence level) for the slopes and intercepts of the regressions. Black circles in the elliptical plots mark the theoretical (intercept = 0, slope = 1) point. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
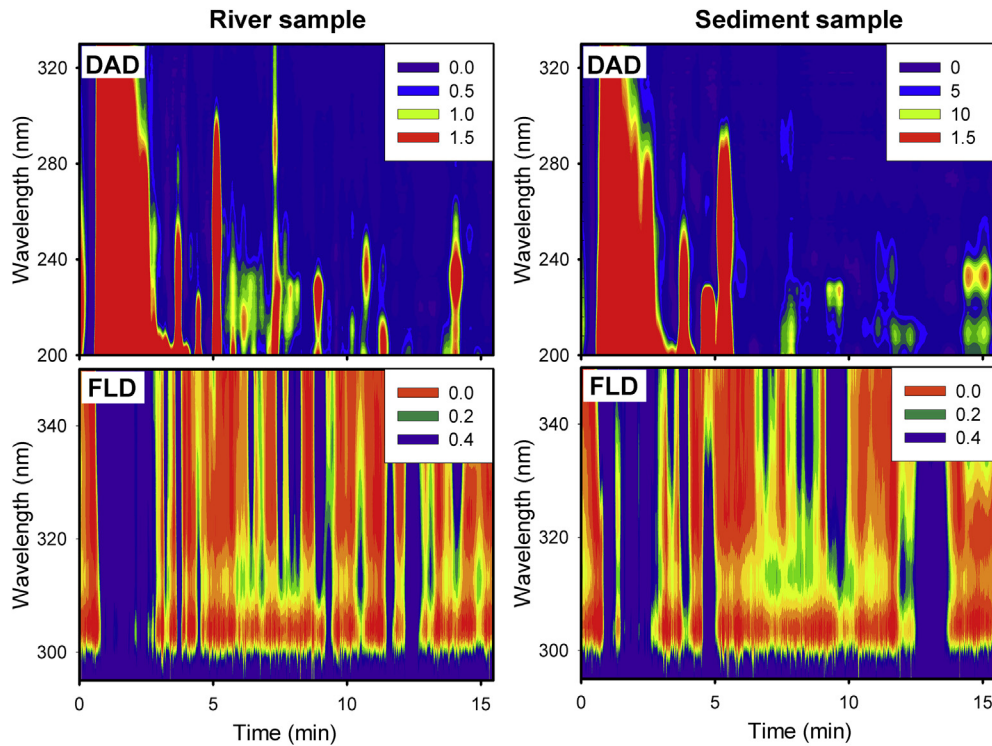
Fig. 5. Two-dimensional contour plots of LC–DAD and FLD matrices for spiked Paraná river water and Carcarañá river sediment samples, in both cases after SPE. The color bars indicate the vertical scales (mAU and UF for DAD and FLD, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
MCR-ALS predicted concentrations (ng L^{-1}) and statistical values in spiked real water samples.[a]

Sample		Androgen/Progestagen[b]				Estrogen[c]						
		NOR	AE	LEV	PROG	E3	E2	EE2	E1	DES	HEX	MEST
UW#1	Taken	24	21	24	19	15	10	15	20	26	10	13
	Found	22(9)	18(1)	27(6)	19 (2)	18 (1)	12(2)	12(1)	24(7)	28(1)	8(1)	13(1)
UW#2	Taken	39	42	39	39	20	19	20	40	42	20	17
	Found	32(8)	36(3)	32(7)	46(2)	19(1)	24(4)	25(2)	39(4)	37(5)	18(2)	15(4)
	RMSEP	5	5	5	5	2	4	4	3	4	2	1
	REP	5	5	5	5	4	8	8	3	4	4	2
	LOD	14	16	14	16	10	9	10	18	16	6	9
MW#1	Taken	24	26	26	20	10	14	10	30	21	10	13
	Found	18(1)	22(4)	27(3)	22(1)	11(3)	15(2)	12(4)	37(4)	27(1)	8(1)	13(4)
MW#2	Taken	49	63	63	54	25	28	25	59	57	20	26
	Found	38(3)	56(2)	67(6)	52(4)	23(7)	26(1)	20(2)	59(7)	53(1)	17(3)	25(2)
MW#3	Taken	98	100	97	88	45	38	50	89	104	44	35
	Found	86(9)	89(7)	89(3)	86(1)	40(8)	43(3)	45(1)	96(1)	108(9)	39(4)	30(1)
	RMSEP	10	8	5	2	3	3	4	6	5	3	3
	REP	10	8	5	2	6	6	8	6	5	6	6
	LOD	14	18	21	16	7	10	8	18	15	6	10
RW#1	Taken	25	32	29	25	10	19	15	20	26	10	17
	Found	25(8)	34(3)	32(2)	23(2)	12(6)	19(1)	12(2)	18(7)	30(1)	11(1)	19(2)
RW#2	Taken	59	47	63	59	25	33	30	55	52	30	31
	Found	52(5)	51(4)	65(2)	59(8)	29(5)	28(1)	31(5)	52(2)	48(2)	28(7)	33(1)
RW#3	Taken	93	95	97	93	46	48	40	99	88	49	52
	Found	105(4)	81(1)	88(2)	95(3)	54(6)	49(6)	35(1)	105(6)	85(3)	46(3)	44(6)
	RMSEP	8	6	6	2	5	3	3	4	4	2	5
	REP	8	6	6	2	10	6	6	4	4	4	10
	LOD	20	24	19	15	6	12	7	16	20	9	16

[a] UW, MW and RW refer to different samples of underground water (Funes, Argentina), mineral water (Mendoza, Argentina) and river water (Paraná river, Argentina), respectively. RMSEP (root-mean-square error of prediction) and LOD (limit of detection calculated according to Olivieri, 2014) are given in ng L^{-1} (pre-concentration factor = 1:500, see text). REP (relative error of prediction) is given in %. The found values are means of duplicates. Standard deviations are given between parentheses.
[b] Measured with DAD.
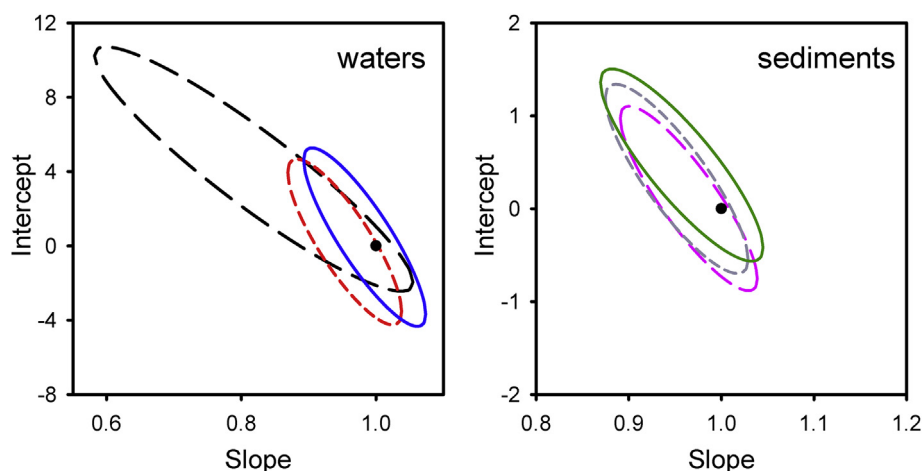[c] Measured with FLD, except E1 and DES (see text).

Fig. 6. Elliptical joint confidence region test at 95% confidence level for the MCR-ALS predicted concentrations of all analytes in water samples [underground (long dashed-black line), mineral (short dashed-red line), river (solid-blue line)] and sediment samples [Carcarañá (long dashed-pink line), Paraná (short dashed-gray line), treatment plant (solid-green line)]. Black circles mark the theoretical (intercept = 0, slope = 1) point. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
MCR-ALS predicted concentrations (ng g^{-1}) and statistical values in spiked real sediment samples.[a]

Sample		Androgen/Progestagen[b]				Estrogen[c]						
		NOR	AE	LEV	PROG	E3	E2	EE2	E1	DES	HEX	MEST
CS#1	Taken	7.4	4.9	6.1	5.0	3.8	2.5	3.1	5.0	6.2	3.7	2.5
	Found	6.5(1)	5.0(2)	7(1)	6(1)	4.0(2)	2.6(4)	3.0(4)	5(1)	5.8(3)	5(1)	3.0(6)
CS#2	Taken	17.3	14.8	15.8	15.0	9.5	8.6	8.0	14.9	15.0	9.3	7.6
	Found	16.7(3)	13(1)	14(1)	14(1)	9.0(1)	9(1)	8.8(6)	13(1)	16.0(2)	8.5(6)	6.8(5)
CS#3	Taken	22.3	21.0	21.8	23.8	11.5	12.4	11.7	24.8	22.5	12.4	12.7
	Found	24(1)	20(3)	20(2)	23.8(2)	11.5(4)	13(1)	10.6(1)	25(1)	22(2)	12(1)	12(1)
	RMSEP	2	3.3	3.8	2.3	0.7	1.3	1.6	1.5	2.3	1.3	1.4
	REP	4	6	8	4	3	5	6	3	4	5	5
	LOD	0.2	0.9	0.6	0.7	0.7	0.7	0.3	0.9	0.6	0.2	0.9
PS#1	Taken	5.0	6.2	8.5	7.5	2.5	4.3	3.7	6.2	7.5	2.5	3.8
	Found	5.0(3)	6.0(5)	9(1)	7.7(2)	2.7(4)	3.9(4)	4.0(1)	7.0(7)	7(1)	2.9(4)	3.8(6)
PS#2	Taken	16.1	16.0	17.0	12.5	7.0	5.6	4.9	17.4	10.0	6.2	6.0
	Found	15(4)	15(1)	17.3(4)	13(1)	7(1)	5(1)	4.4(4)	17(1)	11(1)	6(1)	5.7(2)
PS#3	Taken	24.8	23.4	24.3	21.3	12.7	11.1	10.5	23.6	22.5	10.5	10.1
	Found	23(1)	21(4)	25(3)	18(1)	12.3(6)	10(1)	12(2)	24.2(1)	23(3)	8(1)	11(1)
	RMSEP	1.8	2.6	1	2.6	0.5	1.3	1.4	1.2	1.6	2	1
	REP	4	5	2	5	2	5	5	2	3	8	4
	LOD	0.4	0.8	0.4	0.4	0.3	0.5	0.4	0.8	0.8	0.1	0.8
TPS#1	Taken	7.4	7.4	9.7	5.0	3.8	2.5	2.5	5.0	5.0	3.7	2.5
	Found	6 (1)	7 (1)	9(1)	5(1)	3.4(1)	2.7(3)	2.2(5)	6(1)	6(1)	4.6(1)	2.6(2)
TPS#2	Taken	14.9	14.8	17.0	12.5	6.4	4.9	6.2	12.4	10.0	6.2	5.1
	Found	15.0(3)	16(3)	17.8(2)	13(1)	6.0(2)	5(1)	6(1)	14(1)	10(3)	6.9(4)	6(1)
TPS#3	Taken	22.3	19.7	24.3	17.5	11.4	9.9	8.6	19.8	17.5	9.9	8.9
	Found	21(1)	18(2)	22(1)	19(1)	10(1)	10.0(4)	8(2)	18(2)	20(4)	10.7(2)	9(1)
	RMSEP	2.3	2.0	3.0	1.4	1.5	0.4	0.7	2.3	2.6	1.7	0.7
	REP	4	4	6	3	6	1	3	5	5	7	3
	LOD	0.8	0.5	0.8	0.8	0.8	0.3	0.3	0.8	0.9	0.3	0.5

[a] CS, PS and TPS refer to different sediment samples from Carcarañá and Paraná rivers and a water treatment plant, respectively. RMSEP and LOD (calculated according to Olivieri, 2014) are given in ng g^{-1} (pre-concentration factor = 1:500, see text). REP is given in %. Standard deviation of duplicates, in the last significant figure, is given between parentheses.
[b] Measured with DAD.
[c] Measured with FLD, except E1 and DES (see text).

signal, σ^2_{ycal} is the variance in calibration concentrations, and SEN is the component sensitivity (Bauza et al., 2012). LODs for the analytes determined by DAD, with an average value of 17 ng L^{-1}, approximately double the LOD values for the analytes quantified by FLD (mean LOD = 9 ng L^{-1}). This fact is ascribed to the different detector sensitivities. As expected, the presence of a significant amount of interferents in a sample, such as a river one, produces a deleterious effect in the calculated LODs.

3.5. Sediment samples

Concentrations of estrogens, progestagens and androgens in river sediments are in the range of a few ng g^{-1} (Besse and Garric, 2009; Streck, 2009; Jenkins et al., 2003), and analytes were assayed at these levels.

The good recoveries and statistical values obtained (Table 4) are indicative of the validity of the method and the effectiveness of the

SPE procedure that enables the quantification at very low analyte levels. As in the case of water samples, the results passed the EJCR test (Fig. 6), demonstrating the accuracy of the employed methodology and how the second-order calibration models the interferences naturally present in the studied complex samples. Regarding this latter issue, it is also remarkable how the amount of organic solvents was decreased using the proposed strategy, in comparison with that currently employed in sample pre-treatments for the analysis of the studied hormones in sediments (Streck, 2009; Liu et al., 2012, 2014; Görög, 2011; Labadie and Hill, 2007; Matić et al., 2014).

4. Conclusions

Eleven sex hormones included in the group of endocrine disruptors have been analyzed by LC-DAD-FLD under an isocratic regime, in a short elution time, and applying a minimal sample pre-treatment. The flexibility of the multivariate algorithm (MCR-ALS) allowed the successful resolution of coeluted peaks belonging to analytes and interferents in challenging scenarios, such as those formed by natural waters and sediments. Since the length of the chromatographic run, the solvent consumption, the waste generation and the operator time are significantly reduced, while the frequency of sample processing is notably increased, the proposed method meets the criteria defined in the framework of green chemistry principles and may allow to substitute more complex analytical methods.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.11.024.

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