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Liquid chromatography with diode array detection and multivariate curve resolution for the selective and sensitive quantification of estrogens in natural waters

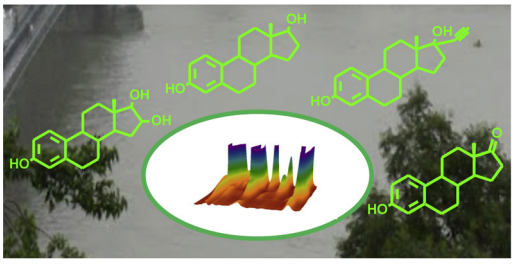
Rocío L. Pérez, Graciela M. Escandar *

Instituto de Química Rosario (CONICET-UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531 (2000), Rosario, Argentina

HIGHLIGHTS

- Potent endocrine disruptors are easily analyzed using non-sophisticated instrumental.
- Selectivity is successfully achieved by applying multivariate curve resolution.
- Quantification in real samples is accomplished using green-chemistry principles.

GRAPHICAL ABSTRACT



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ABSTRACT

Following the green analytical chemistry principles, an efficient strategy involving second-order data provided by liquid chromatography (LC) with diode array detection (DAD) was applied for the simultaneous determination of estriol, 17β-estradiol, 17α-ethinylestradiol and estrone in natural water samples. After a simple pre-concentration step, LC–DAD matrix data were rapidly obtained (in less than 5 min) with a chromatographic system operating isocratically. Applying a second-order calibration algorithm based on multivariate curve resolution with alternating least-squares (MCR-ALS), successful resolution was achieved in the presence of sample constituents that strongly coelute with the analytes. The flexibility of this multivariate model allowed the quantification of the four estrogens in tap, mineral, underground and river water samples. Limits of detection in the range between 3 and 13 ng L^{-1}, and relative prediction errors from 2 to 11% were achieved.

Abbreviations: ANDR, Androgens; BPA, Bisphenol A; CF, Cigarette filter; CORT, Corticosteroids; CP-CPE, Co-precipitation assisted cloud point extraction; DAD, Diode array detector; DERIV, Derivatization; DES, Diethylstilbestrol; DHS, Dihydrostilbestrol; DIS, Dienestrol; DSW, Discharging sites water; DLLME, Dispersive liquid–liquid microextraction; ECF, Estrogen conjugated forms; ENNFM, Electrospun nylon6 nanofibrous membrane; FD, Fluorescence detector; FW, Fishpond water; GC, Gas chromatography; HF-MMLLE, Hollow-fiber microporous membrana liquid–liquid extraction; LC, Liquid chromatography; LVI, Large volume injection; LOD, Limit of detection; LOQ, Limit of quantification; LW, Lake water; MES, Mestranol; MIP, Molecularly imprinted polymer; MM-SPE-MPS, Magnetic-mediated solid-phase extraction micro-particle sorbent; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry; MW, Mineral water; OP, Octylphenol; PPs, Pharmaceutical products; PROG, Progestagens; RW, River water; SBSE, Stir bar sorptive extraction; SPE, Solid-phase extraction; SPW, Spring water; SW, Surface water; SWW, Sewage water; TW, Tap water; UPLC, Ultra performance liquid chromatography; US, Ultrasonication; UW, Underground water; W, Water; WW, Wastewater; WWTPI, Wastewater treatment plant influent; WWTPE, Wastewater treatment plant effluent.

* Corresponding author. Tel.: +54 341 4372704; fax: +54 341 4372704.
E-mail addresses: escandar@iquir-conicet.gov.ar, gmescandar@hotmail.com (G.M. Escandar).

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1. Introduction

Estrogens are steroidal hormones which play an important role in human physiology, including, among others, reproductive female functions, modulation of tissues growth and bone integrity [1]. The three major naturally occurring estrogens 17β-estradiol (E2), estriol (E3), estrone (E1), and the synthetic estrogen 17α-ethynylestradiol (EE2), widely used in contraceptive pills, are the main contributors to the total estrogenicity in waterways [2]. In fact, active estrogen forms are constantly excreted into the aquatic environment and may cause serious health effects in animals and humans, especially in regards to reproduction [3,4]. Since estrogens are the most potent endocrine disrupting compounds (EDCs) which, in turn, are defined as chemicals that may negatively interfere with the endocrine system of humans and wildlife [5], it is not surprising that continuous efforts are devoted to find sensitive and selective methods for their quantification in natural samples.

Complete overviews on the development of the analysis of steroidal hormones in environmental matrices can be found in the literature [2,6–9]. As indicated in the latter works, liquid chromatography (LC) and gas chromatography (GC) followed by detection with mass spectrometry (MS) or tandem MS are the most employed analytical tools to determine estrogens and other EDCs in many different water sources. However, this instrumental is sophisticated and usually requires important capital investment and personnel training. In addition, because of the complexity of certain environmental matrices, a great effort must be devoted to sample preparation, with the additional risk of loss of analytes during extensive extraction and clean up steps [2].

In such situations, multivariate data analysis can be used for improving the selectivity of data collected in less expensive equipment by mathematical means. Specifically, multi-way calibration based on higher-order data (e.g., second-order LC-diode array detection or LC–DAD data) allows the prediction of analyte concentrations in samples containing potential interferences. This useful property, named the "second-order advantage" [10,11], avoids the requirement of interference removal, with the concomitant saving of experimental work and analysis time. Further, toxic organic solvents frequently used for clean up procedures are prevented.

As part of a program devoted to the development of high performance methods within the framework of green chemistry principles [12,13], the use of isocratic LC–DAD data coupled to second-order multivariate calibration, was proposed as a useful approach for rapid and selective detection of estrogens. The LC–DAD matrix data were obtained in short times and using minimal solvent volumes. In the first phase, determinations were carried out in solutions containing the studied estrogens and additional compounds selected as potential interferences. In the second step, the proposed methodology was applied to real samples.

Two issues had to be taken into account when choosing the appropriate algorithm to process the present data: (1) component profiles in the elution time mode usually change in shape and/or position from sample to sample, and (2) the absorption spectra of the studied analytes are very similar. These problems were overcome applying the so-called extended multivariate curve resolution-alternating least-squares (MCR-ALS) algorithm [14], using specific strategies which will be discussed below. It is important to remark that this algorithm has been proposed for handling different types of chromatographic challenges [15,16] and, in the present report, it was successfully used for improving both the sensitivity and selectivity of the applied chromatographic method.

2. Experimental

2.1. Instrumentation

Chromatographic runs were performed on an HP 1200 liquid chromatography (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump, a manual injector fitted with a 50 μL loop and a diode array UV–visible detector set at a wavelength range from 200 to 330 nm. Three C18 chromatographic columns provided by Agilent Technologies (Santa Clara, CS, USA) were checked: Zorbax Eclipse XDB (4.6 mm × 150 mm, 5 μm particle size), Poroshell 120 EC (4.6 mm × 100 mm, 2.7 μm particle size), and Poroshell 120 EC (4.6 mm × 50 mm, 2.7 μm particle size). The data were collected using the software HP ChemStation for LC Rev.HP 1990–1997.

2.2. Reagents and solutions

All reagents were of high-purity grade and used as received. Estriol, 17β-estradiol, 17α-ethynylestradiol, estrone, naproxen (NX), drospirenone (DRSP), norethisterone acetate (NETA), androstenedione (AED), and diazepam (DZM) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Bedford, USA).

Methanol stock solutions of estrogens and potential interferents were prepared and stored in dark flasks at 4 °C. A set of five calibration solutions by duplicate (10 samples) containing E3, E2, EE2 and E1, each equally spaced in the range 0–110 ng mL^{-1}, were prepared by measuring appropriate aliquots of standard solutions, placing them in 2.00 mL volumetric flasks, evaporating the solvent with a nitrogen stream, and completing to the mark with the solvent mixture used as mobile phase. A test set of additional 19 samples, containing the four analytes and also NX, DRSP, NETA, AED, and DZM, were similarly prepared. The concentrations of each potential interferent ranged between 70 and 340 ng mL^{-1}, and were randomly selected.

2.3. Real samples

Because the evaluated water samples (tap, mineral, underground and river waters) did not contain the studied estrogens at levels higher than the attained detection limits, a recovery study was carried out by spiking them with standard solutions of E3, E2, EE2 and E1, obtaining concentration levels in the range 10–100 ng L^{-1}. These water samples were prepared in duplicate and, with the exception of river water, they underwent no previous treatment. River water was collected from Paraná River (Rosario, Argentina) in a 4 L amber glass bottle rinsed with methanol and Milli-Q water, stored at 4 °C immediately after sampling, and analyzed as soon as possible (within 48 h after collection) in order to avoid addition of chemical preservatives. River samples were filtered twice prior to injection: first through a paper filter and then through a cellulose acetate 0.2 μm pore size filter.

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 250 mL of the sample was carried out in approximately 12 min per sample. This flow rate is in the optimum range for maximum breakthrough volume (10–30 mL min^{-1}) [17]. The retained estrogens were eluted with methanol, and this solvent was evaporated with a nitrogen stream. Then, the solutions were reconstituted with 0.200 mL of mobile phase and subjected to the same chromatographic analysis as the test samples. In this way, the preconcentration factor was 2500.

2.4. LC–DAD procedure

The data matrices were collected from 0 to 4.5 min each 1.8 s in the elution time axis, at wavelengths from 200 to 330 nm each 1 nm. The slit width was 1 nm. The LC–DAD matrices of size 149 × 131 (time and spectral data points, respectively) were saved in ASCII format, and transferred to a PC for subsequent manipulation. The mobile phase used for all chromatographic runs 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.

2.5. Software

The data were handled using the MATLAB computer environment [18]. The calculations involving MCR-ALS have been made using mvc2_gui, a MATLAB graphical interface toolbox which is a new version of that already reported in the literature [19], freely available at www.iquir-conicet.gov.ar/descargas/mvc2.rar.

3. Theory

The MCR-ALS algorithm has been discussed in detail [14], and thus only a brief description is presented here. In this algorithm, an augmented data matrix is created from the test data matrices and the calibration data matrices. These individual matrices are of size $J \times K$, where J is the number of elution times (number of rows of each data matrix) and K the number of emission wavelengths (number of columns of each data matrix). Augmentation can be performed either column-wise or row-wise, depending on the type of experiment being analyzed [20]. In the presently studied case, the augmentation was implemented column-wise, i.e., in the elution time direction, because in this way the chemical rank of the augmented matrix is better preserved.

In the column-wise augmentation mode, the bilinear decomposition of the augmented matrix is performed according to the expression:

$$D = CS^T + E \quad (1)$$

where the columns of D contain the chromatograms measured at J times for $(I_{cal} + 1)$ different samples at K wavelengths, the columns of C contain the augmented elution time profiles of the intervening species, the columns of S their related spectra, and E is a matrix of residuals not fitted by the model. The sizes of these matrices are D, $J(I_{cal} + 1) \times K$, C, $J(I_{cal} + 1) \times N$, S, $K \times N$, E, $J(I_{cal} + 1) \times K$ (N is the number of responsive components). As can be observed, D contains data for the I_{cal} calibration samples and for a given test sample. Decomposition of D is achieved by iterative least-squares minimization of the residuals contained in E, under suitable constraining conditions such as non-negativity, unimodality, correspondence, selectivity, trilinearity, closure, etc. [20].

MCR-ALS requires initialization with parameters as close as possible to the final results. For example, the species spectra can be supplied, as obtained from either pure analyte standards or estimated from the analysis of the so-called 'purest' spectra [21–23], applying a multivariate algorithm which extracts pure component spectra from a series of spectra of mixtures of varying composition [21]. Another option is to provide estimated elution time profiles, as obtained from procedures such as evolving factor analysis (EFA) [24]. Specific constraints and initialization applied in the present case will be explained below.

After MCR-ALS decomposition of D, concentration information contained in the elution profiles (C matrix) can be used for quantitative predictions, by first defining the analyte score as the area under the profile for the ith sample:

$$a(i,n) = \sum_{j=1+(i-1)J}^{iJ} C(j,n) \quad (2)$$

where $a(i,n)$ is the score for the analyte n in the sample i, and $C(j,n)$ is the element of the analyte profile in the augmented mode. The analyte scores in the calibration samples are employed to build a pseudo-univariate calibration graph against the nominal analyte concentrations, predicting the concentration in the test samples by interpolation of the test sample score.

4. Results and discussion

4.1. Selection of optimal experimental conditions

In order to achieve the resolution of the studied estrogens in the shortest possible time and using the least amount of organic solvent, chromatographic conditions were optimized. According to previous experience related to the chromatographic determination of estrogens [25,26], mobile phases containing different ratios of acetonitrile and water were tested, and a mobile phase constituted by acetonitrile–water in a 50:50 ratio provided the best resolved peaks. Thus the latter mobile phase was used in all runs.

For the three C18 chromatographic columns of 50, 100 and 150 mm length packed with particles of 2.7, 2.7 and 5 μm average diameter, respectively, different loop volumes (5, 20, 50 and 100 μL) and flow rates in the range 0.8–1.5 mL min^{-1} were probed. It was corroborated that the 100 mm column packed with 2.7 μm particles, a 50 μL loop sample and a flow rate of 1 mL min^{-1} produced better signals. The pH values of the sample solutions were approximately neutral (the pH was not adjusted).

A model system of the four analytes prepared in a mobile phase solution was tested using the working conditions summarized in Table 1. Estrogens peaks were resolved in less than 4.5 min using an isocratic regime, the elution order being E3, E2, EE2 and E1.

4.2. Multivariate calibration results

In real samples, the simultaneous presence of additional matrix constituents, which overlap both in the spectral and time modes with the analytes, precludes the estrogens quantification through classical zeroth-order calibration. In this latter case, it is highly convenient to use second-order calibration with suitable algorithms for the quantitation of the analytes, because of the need of achieving the second-order advantage [10,11]. However, an additional limitation inherent to chromatographic second-order data is the lack of repeatability in the elution time profiles between successive runs, which prevents the use of algorithms requiring that the data show the trilinearity property [27]. In this regard, MCR-ALS was selected for data processing because this algorithm achieves the second-order advantage and has the additional benefit of not requiring that a given component shows the same chromatographic profile in each experimental run [28].

Table 1
Instrumental and chemical parameters.

	Values/reagents
Mobile phase	Acetonitrile/water (50:50, v/v)
Column	Poroshell 120 EC (4.6 mm × 100 mm, 2.7 μm particle size)
Volumetric flow-rate (mL min^{-1})	1.0
Temperature	Room-temperature
Injection volume (μL)	50
Time range (min)	From 0 to 4.5
Wavelength range (nm)	From 200 to 330
Calibration range (ng mL^{-1})	From 0 to 110

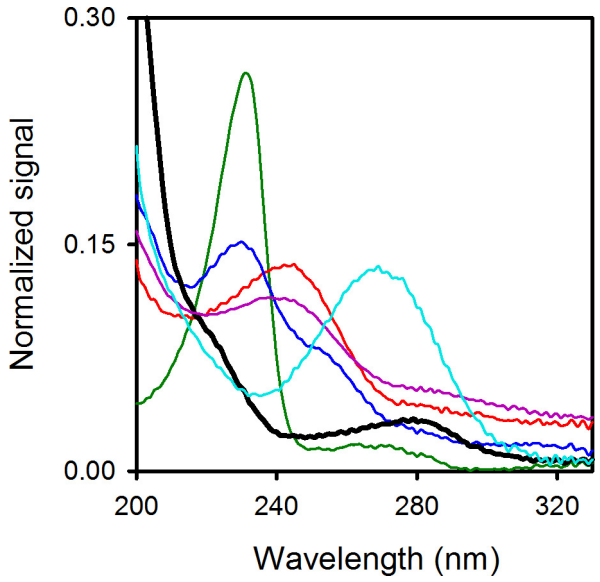

Fig. 1. Normalized absorption spectra in acetonitrile-water (50:50, v/v) for the assayed estrogens (thick black line), NX (green line), DZM (blue line), DRSP (cyan line), NETA (violet line), and AED (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In a first stage, samples containing the studied analytes and potential interferences were processed. Real samples containing their own constituents were then studied.

4.2.1. Synthetic samples

With the purpose of mimicking a real situation, test samples were prepared containing the estrogens solutions and also foreign compounds which could be concomitantly present in natural waters. It was verified that emerging pollutants such as naproxen (an anti-inflammatory drug), drospirenone and norethisterone acetate (two progestins), androstenedione (a sex hormone precursor), and diazepam (a psychiatric drug) coelute with the analytes under the established working conditions, and also strongly overlap their spectral signals (Fig. 1). Therefore, these compounds were selected as potential interferents.

Fig. 2 shows the contour plots for typical LC–DAD matrices recorded for a calibration and for a test sample, where the high

Table 2
Selected chromatographic/spectral ranges used for MCR-ALS data processing.

Analyte	Time (min)	Wavelength (nm)
E3	0.82–1.19	200–330
E2	2.10–2.86	200–330
EE2	2.86–3.40	200–330
E1	3.40–4.07	200–330

complexity of the analytical problem under study can be appreciated.

Because the four studied estrogens have very similar UV spectral profiles (see Fig. 1), it was not possible to perform MCR-ALS analysis with matrix augmentation in the temporal direction (i.e., column-wise) when working with the full chromatogram (e.g., involving the complete elution time range). This would lead to almost zero spectral selectivity, making it difficult the decomposition of the augmented matrix. An alternative in this case is to augment the matrices in the spectral direction (i.e., row-wise) [29]. However, due to elution time shifts and band shape changes among calibration and test samples, matrix augmentation in the spectral direction is also inconvenient. Hence, MCR-ALS was applied by column-wise augmentation of chromatographic data matrices, but dividing the elution time axis in four time regions, each one including a single analyte (see Table 2 and Fig. 2A).

In this latter case, the chemical rank would be equal to the mathematical pseudorank, because the component spectra do not change from sample to sample. Thus, data processing comprised the building of augmented column-wise D matrices containing, for each time region and in the whole wavelength range, data for each of the analyzed samples and for the calibration samples.

Before starting MCR-ALS resolution, the estimation of the number of spectrally active components in each D data matrix was made from the plot of singular values as a function of a trial number of components, locating a number for which the plot stabilizes. The latter number is initially employed for MCR-ALS analysis, and is afterwards refined (increased or decreased) until an appropriate solution is found, with a reasonable least-squares fit and physically recognizable profiles. For a given number of responsive components, their spectra were then estimated from the analysis of the so-called purest variables [21]. The profiles provided by the latter analysis were suitable to perform the

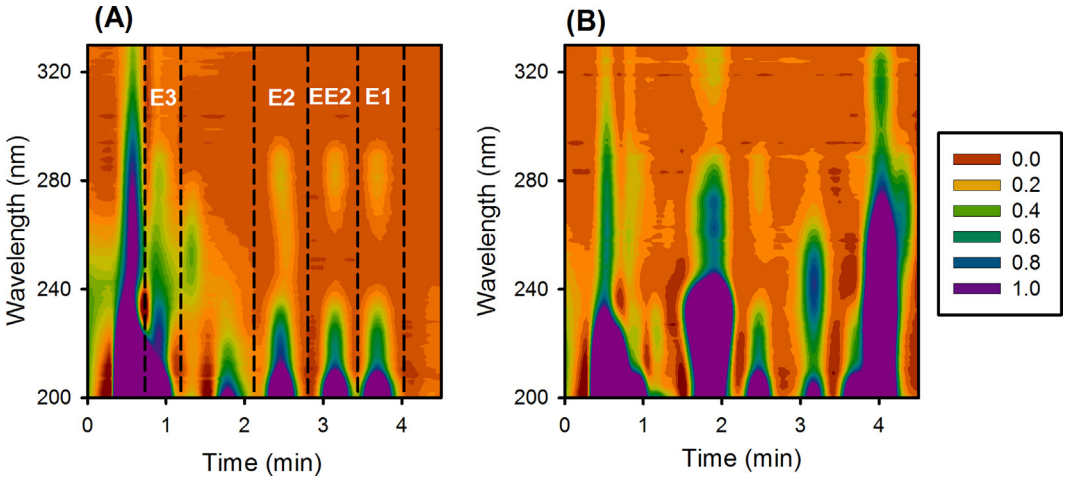

Fig. 2. Two-dimensional contour plots of LC–DAD matrices for samples only containing the studied estrogens (A), and in the presence of NX, DZM, DRSP, NETA and ADE as interferences (B). Dashed lines in (A) delimit the selected chromatographic/spectral regions used for data processing of each analyte, as indicated. Concentrations are as follows (all in ng mL^{-1}): (A) E3, 102; E2, 110; EE2, 109; E1, 101. (B) E3, 66; E2, 83; EE2, 38; E1, 96; NX, 252; DZM, 308; DRSP, 78; NETA, 60 and AED, 70.

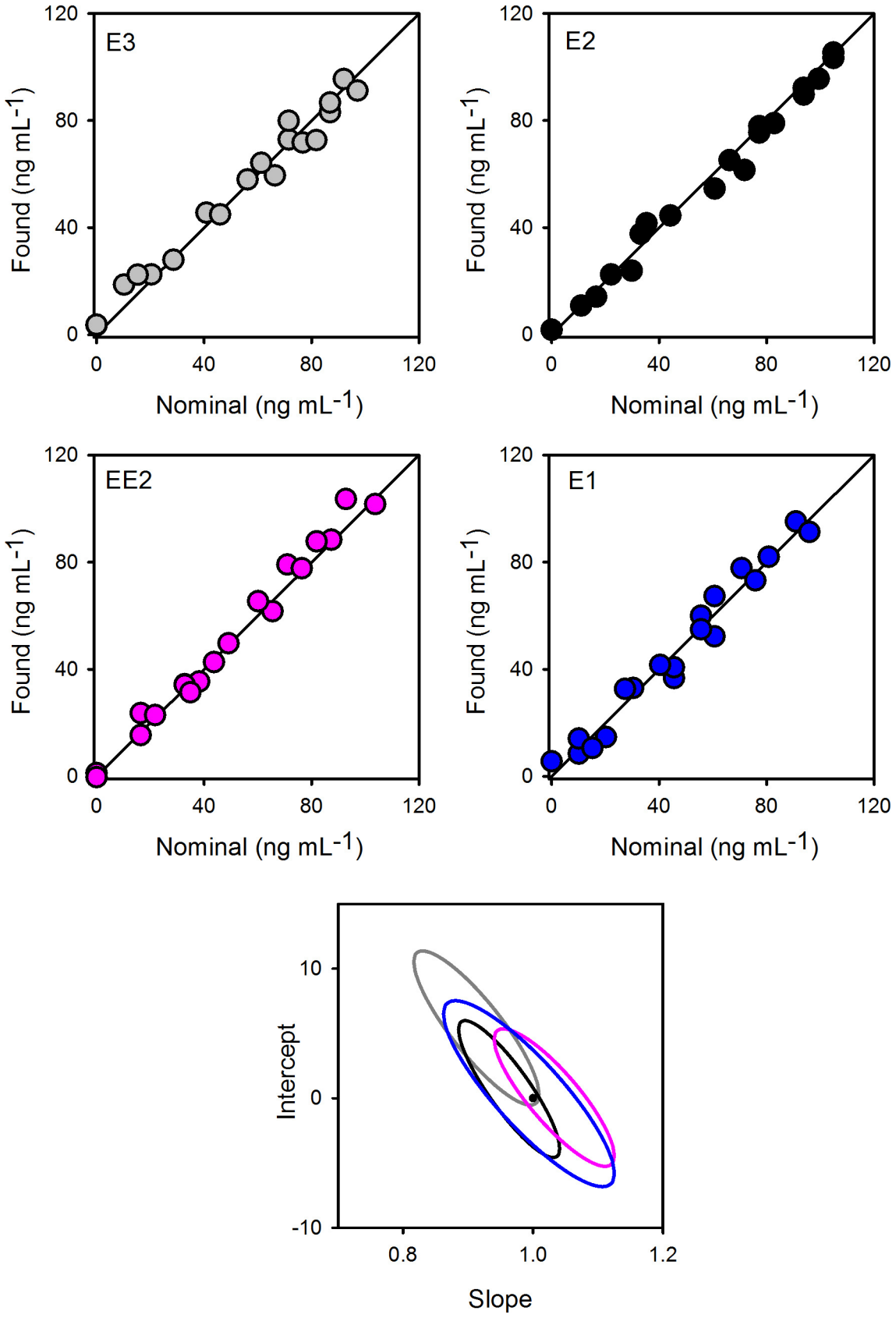

Fig. 3. Plots of E3 (gray), E2 (black), EE2 (pink), and E1 (blue) predicted concentrations as a function of the nominal values in test samples (as indicated), and elliptical joint regions (at 95% confidence level) for the slopes and intercepts of the regressions for the corresponding predictions. The black dot in the elliptical plots marks 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 3
Statistical results for the studied estrogens in samples with NX, DZM, DRSP, NETA and ADE as potential interferents (test set) and in spiked water samples using LC–DAD matrices and MCR-ALS.

	E3	E2	EE2	E1
Synthetic test set				
SEL	0.93	0.77	0.90	0.72
γ (mL ng^{-1})	4.3	4.7	5.0	4.8
LOD (ng mL^{-1})	14	10	10	8
LOQ (ng mL^{-1})	42	30	30	24
RMSEP (ng mL^{-1})	5.1	4.1	4.4	5.1
REP (%)	10	7.4	8.0	10
Tap water[a]				
SEL	0.56	0.85	0.83	0.81
γ (mL ng^{-1})	1000	1800	3300	3400
LOD (ng mL^{-1})	0.012	0.012	0.010	0.006
LOQ (ng mL^{-1})	0.036	0.036	0.030	0.018
RMSEP (ng mL^{-1})	0.005	0.003	0.005	0.001
REP (%)	11	7	11	2
Mineral water[a]				
SEL	0.90	0.67	0.86	1.0
γ (mL ng^{-1})	4400	4700	4600	4100
LOD (ng mL^{-1})	0.013	0.008	0.008	0.008
LOQ (ng mL^{-1})	0.039	0.024	0.024	0.024
RMSEP (ng mL^{-1})	0.003	0.002	0.003	0.001
REP (%)	8	5	6	3
Underground water[a]				
SEL	0.47	0.53	0.88	0.57
γ (mL ng^{-1})	1300	3400	4400	4800
LOD (ng mL^{-1})	0.003	0.007	0.006	0.007
LOQ (ng mL^{-1})	0.009	0.021	0.018	0.021
RMSEP (ng mL^{-1})	0.001	0.002	0.003	0.001
REP (%)	2	5	8	3
River water[a]				
SEL	0.60	0.83	0.68	0.54
γ (mL ng^{-1})	3700	4000	3600	5600
LOD (ng mL^{-1})	0.010	0.008	0.011	0.010
LOQ (ng mL^{-1})	0.030	0.024	0.033	0.030
RMSEP (ng mL^{-1})	0.002	0.001	0.002	0.002
REP (%)	5	3	6	6

SEL: selectivity calculated according to Ref. [32]; γ: analytical sensitivity; LOD: limit of detection calculated according to ref. [29]; LOQ: limit of quantfication calculated as LOD × 3; RMSEP: root-mean-square error of prediction; REP: relative error of prediction.

[a] The results refer to water samples before SPE. For comparison with the test samples, values for water samples are given in ng mL^{-1}.

resolution and, therefore, it was not necessary to include reference spectra for the analyte as initial estimates for MCR-ALS.

In order to drive the iterative procedure to chemically interpretable solutions, non-negativity constraints in both modes, correspondence restriction, and unimodality constraint in the temporal mode were applied. The selected MCR convergence criterion was 0.1% (relative change in fit for successive iterations) and the maximum number of iterations was 2500. However, convergence was achieved after less than 10 iterations in most of the evaluated samples.

Fig. 3 shows the prediction results corresponding to the application of MCR-ALS to a set of 19 test samples. As can be observed, the predictions for the four estrogens are in good agreement with the corresponding nominal values. If the elliptical joint confidence region (EJCR) [30] is analyzed for the slope and intercept of the above plot, we conclude that ellipse includes the theoretically expected values of (1,0), indicating the accuracy of the used methodology. The good recoveries obtained after the application of MCR-ALS suggest that interacting background effects, which could be present in chromatographic analysis of complex matrices [31], are not significant and, therefore, the use of external calibration was an adequate option. The statistical results are completed with the values shown in Table 3.

The relative errors of prediction (all below 15%) indicate good precision. The obtained values of both limits of detection (LODs) and quantification (LOQs), in the order of parts-per-billion, demonstrate the positive effect of second-order data in the sensitivity of the method [29]. However, considering the estrogen levels which can be found in water samples in parts-per-trillion (see below), it is evident that a pre-concentration step is required for the subsequently evaluated real systems.

4.2.2. Real water samples

With the purpose of testing the present method in real samples and demonstrating its ability of overcoming the interference from background constituents, waters from different origins were analysed.

In water bodies, estrogens are detected in a wide range of concentrations, generally in the order of parts-per-trillion levels [7]. Therefore, the sensitivity of the method was increased through a pre-concentration step employing C18 membrane-SPE. It is necessary to point out that the selection of C18 membranes is based on our excellent experience with this solid-support as extractor of low-polarity compounds, such as the studied analytes [28,33]. Additionally, these membranes are easily and rapidly conditioned, decreasing the laboratorist effort. Because the selectivity between the analytes and interferences is provided by the chemometric tool, the complete physical separation of target analytes from the matrix constituents is not required, as in traditional extraction techniques.

Fig. 4 shows contour plots of LC–DAD matrices corresponding to different real water samples after the SPE procedure. As expected, the C18 membrane also retains other matrix constituents which can interfere, as in the present case, co-eluting with the estrogens and overlapping their absorption spectra. For applying classical zeroth-order calibration, these interferences should be completely removed before the quantification is performed. However, this fact does not represent a problem when using an appropriate second-order calibration approach.

The chromatographic and spectral regions processed for each analyte were the same as those used for test samples. Table 4 shows the results of the recovery study performed by spiking water samples with appropriate amounts of estrogens, in duplicate, at three different concentration levels. The average recovery of the four estrogens in each type of water at the three different fortification levels was tested for significance by using the Student *t*-test: the null hypothesis corresponds to the recovery of 100% [30]. The *t* values obtained for $n-1$ degrees of freedom (where n is the number of evaluated levels) at a 95% of significance compare favorably with the corresponding tabulated value [t_{crit} $_{(0.05,2)}$ = 4.30], suggesting that the proposed method is appropriate for the determination of the studied compounds.

The outstanding results obtained after MCR-ALS was applied to the data suggest that the method can overcome the problem of the presence of unexpected interferents from the background of the real samples. As an example, Fig. 5 shows the profiles retrieved by MCR-ALS in both spectral and temporal modes for a real matrix (underground water in this example) added with the analytes. From top to bottom, each pair of plots in Fig. 5 shows the retrieved spectral and augmented time profiles for the studied analytes (these augmented time profiles contain successive sub-profiles for the unknown and calibration samples). For each analyte, the specific elution time region was the same as for the test samples, and repeats itself in each sub-profile of the augmented time profile. It can be concluded that, although interferences are present in all regions, the spectra are correctly distinguished, and the chromatographic bands are recognized as belonging to the corresponding estrogen and background (present in water and calibration samples) or interferences (only present in the real sample).

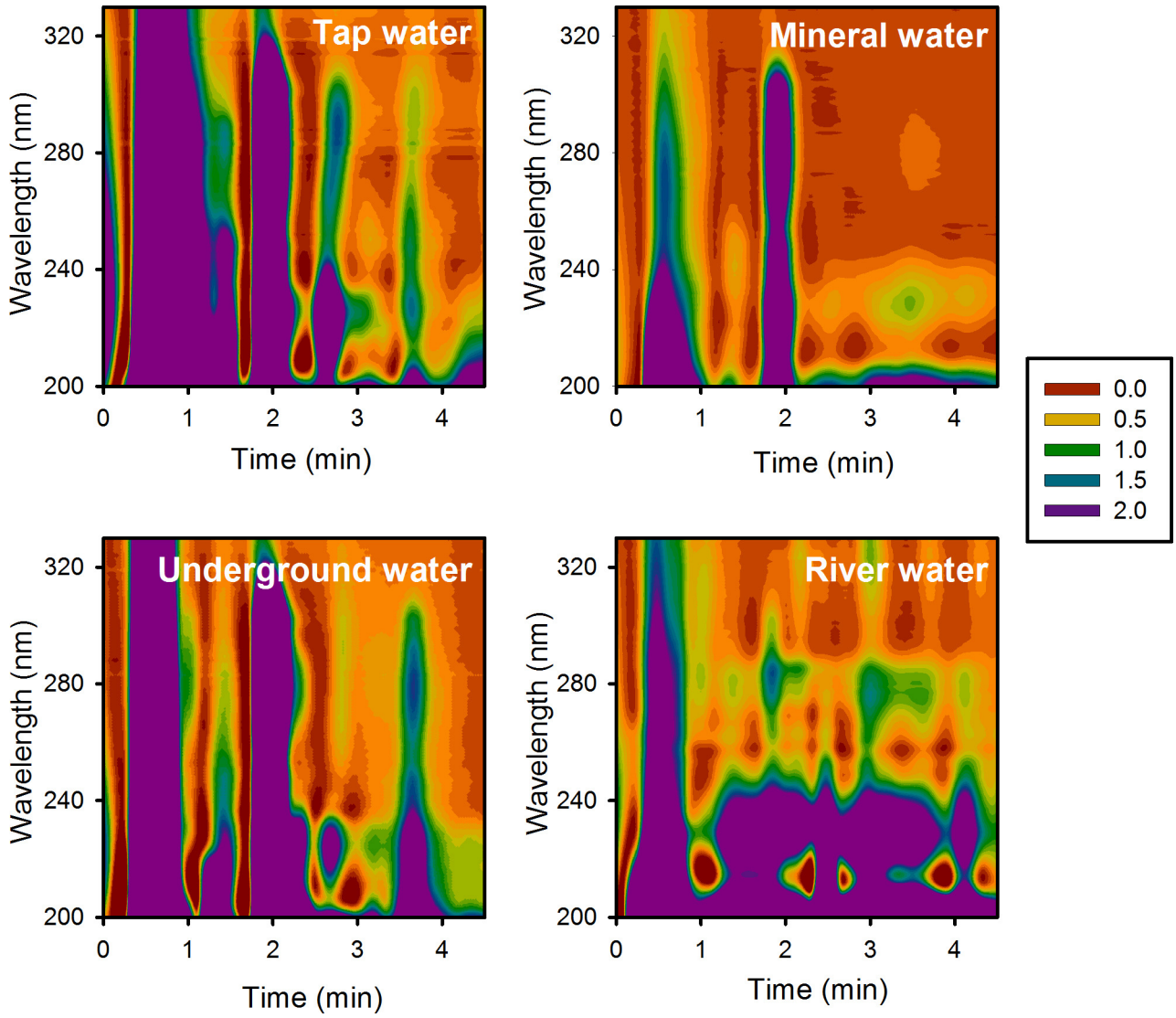

Fig. 4. Two-dimensional contour plots of LC–DAD matrices for different water samples, after SPE, spiked with the studied analytes. Original concentrations are as follows (all in ng L^{-1}): E3, 53; E2, 51; EE2, 50; E1, 53 (tap water); E3, 42; E2, 35; EE2, 49; E1, 41 (mineral water); E3, 53; E2, 42; EE2, 80; E1, 74 (underground water); E3, 46; E2, 40; EE2, 41; E1, 40 (river water).

Table 4
Recovery study for the studied estrogens in spiked water samples using MCR-ALS[a].

	E3				E2				EE2				E1			
	Taken	Found[b]	*R*	*t*[c]	Taken	Found[b]	*R*	*t*[c]	Taken	Found[b]	*R*	*t*[c]	Taken	Found[b]	*R*	*t*[c]
TW[d]	105	102(2)	97		103	107(1)	104		100	114(2)	114		105	99(1)	94	
	53	54(7)	102		51	49(3)	96		50	46(5)	92		52	51(3)	98	
	25	27(1)	108	0.69	22	25(4)	114	0.96	26	24(4)	92	0.14	24	23(2)	96	3.46
MW[e]	18	22(1)	122		18	18(2)	100		18	15(2)	83		16	15(2)	94	
	13	13(2)	100		12	13(1)	108		13	13(1)	100		10	9(5)	94	
	42	43(7)	102	1.15	35	34(4)	97	0.58	49	51(6)	104	0.63	40	41(6)	103	0.99
UW[f]	53	47(7)	100		41	43(4)	104		80	81(3)	101		74	73(4)	99	
	33	33(5)	100		20	19(6)	95		35	33(3)	94		16	15(2)	94	
	14	15(2)	107	0.19	12	14(2)	117	0.79	15	19(1)	126	0.76	11	12(1)	109	0.22
RW[g]	21	23(1)	110		16	17(4)	106		22	22(3)	100		20	17(1)	85	
	46	48(3)	104		40	44(7)	110		41	41(1)	100		40	40(3)	100	
	84	84(5)	100	1.73	80	82(3)	103	2.60	82	87(5)	106	1.15	79	81(2)	103	0.69

[a] Concentrations are given in ng L^{-1} and recoveries (*R*) in percentage.
[b] Means of duplicates. Standard deviation between parentheses.
[c] Calculated student *t* for the average recovery. The critical *t* value for $n-1$ degrees of freedom at a 95% significance level is $t_{crit(0.05,2)} = 4.30$ (see text).
[d] TW, tap water from Rosario city (Santa Fe, Argentina).
[e] MW, mineral water from Villavicencio hills (Mendoza, Argentina).
[f] UW, underground water from Funes City (Santa Fe, Argentina).
[g] RW, river water from Paraná river (Argentina).

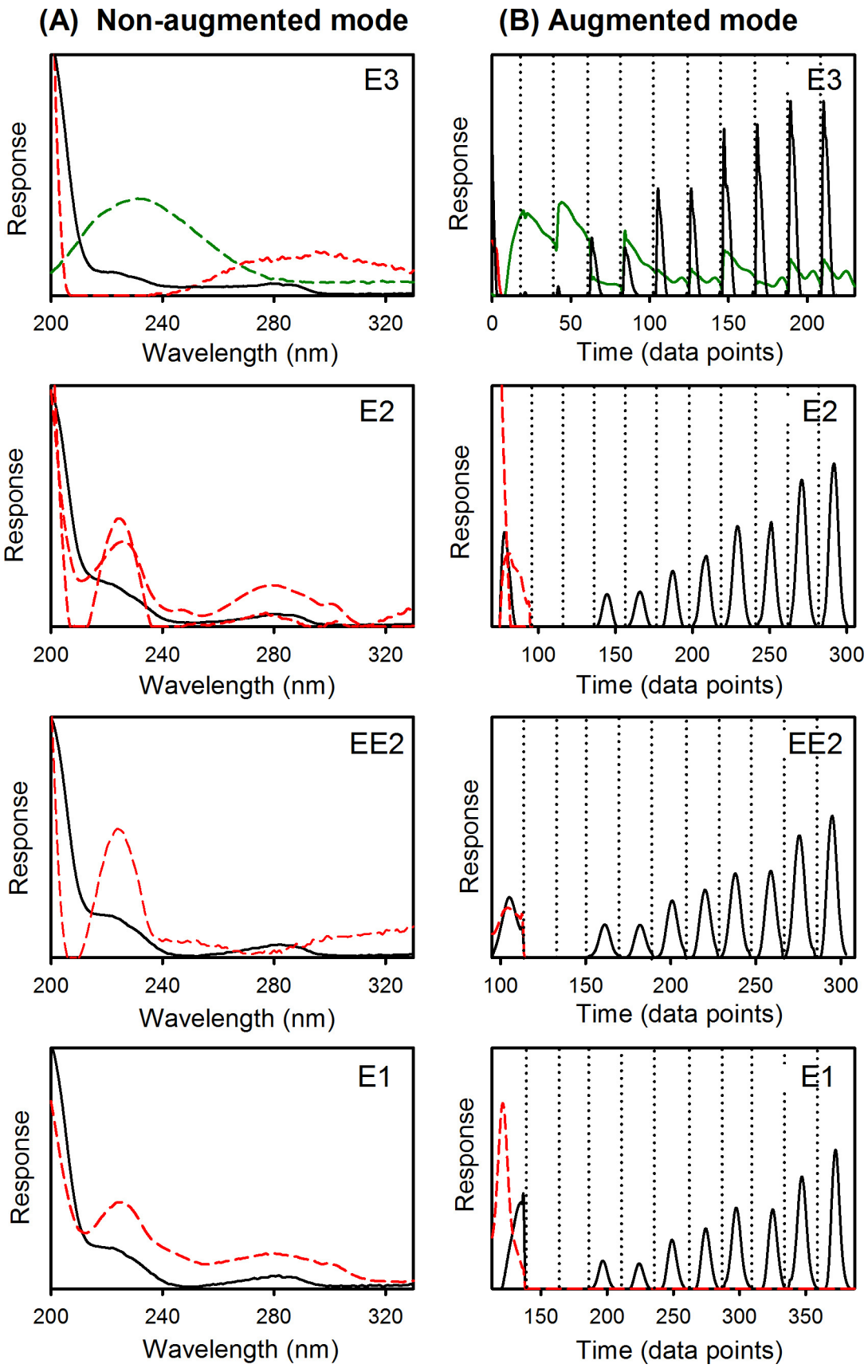

Fig. 5. Profiles retrieved by MCR-ALS when processing an underground water sample spiked with the four estrogens. (A) Spectral profiles in the selected E3, E2, EE2 and E1 chromatographic regions, as indicated. (B) The corresponding time profiles (the dotted vertical lines separate, from left to right, the studied sample and the successive calibration samples). In all plots, the solid black line indicates estrogen, and dashed green and red lines indicate background and interferents, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Analytical performance of selected chromatographic methods reported in the last years for estrogens in natural waters.

Sample pretreatment	Method	Estrogen	Other	LOD[a]	Sample	Refs.
Gas chromatography						
SPE (C18)	LVI–GC–MS	E1, E2, EE2		0.041, E1; 0.046, E2; 0.031, EE2	SW, WW	[34]
HF-MMLLE-deriv	GC–MS	E1, E2, EE2		3, E1; 1.6, E2; 10, EE2	TW, SWW	[35]
SPE (Oasis HLB)-deriv	GC–MS/MS	E1, 17α- and 17β-E2, E3, EE2, MES	andr	0.7–3	SW, treated effluents	[36]
Liquid chromatography–mass spectrometry						
SPE (Strata)	LC–MS/MS	E1, 17α-E2, 17β-E2, E3, EE2	andr, prog	0.02, E1, 0.01, 17β-E2; 0.03, 17α-E2, 0.03, E3; 0.2, EE2	SW, UW	[37]
Deriv-SPE (Oasis HLB)	LC–MS/MS	E1, E2, E3	ECF	0.038,E1; 0.13, E2; 0.11, E3	RW	[38]
SPE (Oasis HLB + Florisil)-deriv	LC–MS/MS	E1, 17α-E2, 17β-E2, E3, EE2		0.07, E1; 0.084, 17α-E2; 0.078, 17β-E2; 0.28, E3; 0.067, EE2	RW	[39]
On line SPE (hypersil gold C18)	LC–LC–MS/MS	E1, E2, E3, EE2	prog	10, E1; 3, E2; 50, E3; 7, EE2	SW, SWW	[40]
SPE (C18 disk)	UPLC–MS/MS	E1, E2, E3, EE2	prog	0.5, E1; 0.6, E2; 1.0, E3; 1.2, EE2	TW, RW, LW, WW	[41]
SPE (Oasis HLB + Florisil)	LC–MS/MS	E1, 17α- and 17β-E2, E3, EE2	ECF	0.4–3[b]	RW, WWTPI, WWTPE	[26]
SPE (Oasis HLB + silica or Florisil)	LC–MS/MS	E1, 17α-E2, 17β-E2	andr, prog, cort	0.008–0.5	RW, DSW, effluents	[42]
MM-SPE-MPS	LC–MS/MS	E1, E2, E3, EE2	BPA	1–36	TW, sea W, SWW	[43]
On line SPE (NG1)	LC–MS/MS	E1, E2, E3, EE2, DES	andr	0.5–2	RW, WWTPI, WWTPE	[44]
US + SPE	LC–MS/MS	E1, E2, E3		0.07–60[c]	Sea W	[45]
Liquid chromatography-spectroscopy						
On line SPE (CF)	LC–UV	E1, E2, E3, DES		16.2, E1; 78.1, E2; 5.6, E3; 0.98 DES	RW, LW, well W	[46]
SPE (MIP)	LC–UV	E1		5.7	Well W, LW	[47]
SPE (ENNFM)	LC–UV	E1, E2, EE2		170, E1; 50, E2; 80, EE2	Natural waters	[48]
DLLME	LC–UV	E1, E2		100, E2; 200, E1	SPW, TW, RW	[49]
DLLME	LC–DAD–FD	E1, E2, E3, EE2, DES		80–500	RW, sea W, WW	[50]
On line SPME	LC–FD	E3, EE2	Prog, BPA	5–30	Natural waters	[51]
CP-CPE	LC–UV	E1, E2, EE2, DES, DHS		200–700	RW, LW	[52]
Coated SBSE	LC–UV	E1, E2, EE2, DES, DIS	BPA, OP	290, E1; 280, E2; 350, EE2; 260, DES; 180, DIS	RW, LW, FW	[53]
SPE (Strata)	LC–UV–FD	E1, E2, EE2	PPs	10–1100[b]	RW, WWTPI, WWTPE	[54]
DLLME	LC–FD	E2, EE2		2.0, E2; 6.5, EE2	TW, SW, WW	[55]
SPE (C18 disk)	LC–DAD–MCR–ALS	E1, E2, E3, EE2		6–10, E1; 7–12, E2; 3–13, E3; 6–11, EE2	TW, MW, UW, RW	This work

[a] For comparison, concentration units were unified to ng L^{-1}.
[b] Limits of quantification.
[c] Levels of measured concentrations.

Table 3 displays the statistical results corresponding to the estrogen determination in real water matrices. These results indicate that neither the selectivity nor the relative error of prediction is significantly affected by the fact that real matrices are being studied. Besides, the analytical sensitivity and limits of detection and quantification reflect the benefits of the preconcentration, and the possibility of determining the studied analytes at part-per-trillion levels. In comparison with the performances of selected chromatographic methods reported in the last years for the determination of estrogens in water samples (Table 5), limits of detection from 0.03 to 10, 0.01 to 36, and 1 to 1100 ng L^{-1} have been found using GC–mass spectrometry, LC–mass spectrometry, and LC–spectroscopic methods, respectively. In the present case, low limit of detections are achieved (LODs = 3–13 ng L^{-1}) applying a non-sophisticated method such as LC–DAD, with a relatively small use of organic solvents and without the need of derivatization as most CG procedures [7] and some LC methods [39].

5. Conclusions

Liquid chromatography-diode array detection associated to multivariate curve resolution-alternating least-squares (MCR-ALS) has demonstrated to be a powerful tool for the determination of estradiol, estriol, estrone and ethynylestradiol in water samples.

Additional properties should be remarked beyond the outstanding sensitivity and selectivity achieved in the present work, which led to limits of detection between 3 and 13 ng L^{-1}, and excellent precision (prediction errors were equal to or below 11%). The use of an appropriate chemometric tool makes it unnecessary to apply extraction and clean up steps for the removal of coeluting compounds, avoiding the use of toxic organic solvents (essential for environmental safety), and saving experimental time and operator effort.

The good quality of the obtained results suggests that the proposed method is appropriate for the rapid analysis of the studied endocrine disrupting agents in natural waters, favorably competing with sophisticated methods usually employed in this type of determinations, which require expensive equipment and derivatization and sample extraction/clean up procedures.

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