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

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7 Rocío L. Pérez, Graciela M. Escandar*

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9 *Instituto de Química Rosario (CONICET-UNR), Facultad de Ciencias*
10 *Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531*
11 *(2000) Rosario, Argentina. E-mail: escandar@iquir-conicet.gov.ar*

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19 * Corresponding author e-mail: escandar@iquir-conicet.gov.ar; phone: +54-341-4372704

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).

Capsule Abstract

Liquid chromatography coupled to chemometrics allows one to selectively and sensitively quantitate eleven endocrine disruptors in challenging scenarios using a green analytical approach

Keywords: High-performance-liquid chromatography; Dual UV-fluorescence detection; Multivariate curve resolution; Endocrine disruptors; Environmental samples.

45 **1. Introduction**

46

47 Natural and synthetic hormones (estrogens, progestagens and androgens),
48 phytoestrogens and some industrial chemical compounds constitute a group of contaminants
49 called endocrine disruptors (EDs) [1]. The presence of EDs in the environment represents a
50 specific pollution threat with potential ecological and human health implications [1,2].

51 Estrogens and progestagens are constantly excreted by humans, reaching the aquatic
52 environment through sewage systems and, therefore, domestic wastewaters are established as
53 a main source of contamination for these EDs [3,4]. Sources of androgens include, in addition
54 to treated domestic wastewater, livestock breeding, pulp mills and degradation of natural
55 phytosterols [5,6].

56 The determination of sexual hormones in aquatic bodies and related environmental
57 samples such as sediments is a very important activity in modern steroid hormone analysis
58 [7]. While numerous reports have been published on the determination of estrogens in
59 environmental waters and, to a lesser extent, sediments, studies on progestagenic and
60 androgenic hormones are scarce [4,5]. Several comprehensive reviews about this subject have
61 been published [3,5,7,8]. Gas chromatography-mass spectrometry (GC-MS), which usually
62 requires a derivatization step, has been progressively replaced by liquid chromatography
63 (LC)-based techniques coupled with MS or tandem MS for quantification of estrogenic,
64 progestagenic and androgenic compounds in complex environmental matrices. The latter
65 techniques offer outstanding sensitivity and selectivity, although they employ sophisticated
66 detectors and strict extraction and clean up processes are mandatory before their application
67 [5].

68 A current trend in environmental analysis is to avoid sample pre-processing steps and
69 long chromatographic runs, exploiting the ability of modern data processing tools for

70 mathematical resolution of coeluting components. Needless to say, analytical methods for
71 pollutants quantification should not contribute with additional contamination. Within the past
72 few years, a new set of methods has arisen, the so-called "green analytical chemistry" (GAC)
73 methods. The driving force has been the need to protect the environment, without negative
74 impact on basic analytical properties [9,10].

75 The main objective of the present work was the development of a GAC method for the
76 analysis of a significant number of sex hormones at part per trillion concentrations in surface
77 and underground waters and sediments. The natural estrogens estriol (E3), 17β-estradiol (E2)
78 and estrone (E1) and the synthetic 17α-ethynylestradiol (EE2) have been previously studied
79 coupling LC-diode-array detection (DAD) data to chemometric analysis [11]. In the present
80 work, single-run dual DAD and fluorescence detection (FLD) are applied for the
81 determination of eleven analytes involving natural (E3, E2, E1) and synthetic [EE2,
82 diethylstilbestrol (DES), hexestrol (HEX), mestranol (MEST)] estrogens, endogenous
83 [progesterone (PROG)] and synthetic [norethisterone (NOR), levonorgestrel (LEV)]
84 progestagens, and a common precursor of male and female sex hormones, androstenedione
85 (AE) (Fig. 1). The dual detection allows us to quantify: (1) estrogens, through the intense
86 fluorescence displayed by most of them in the employed mobile phase, and (2) the remaining
87 non-fluorescent hormones by their UV absorption properties. The benefits obtained by
88 combining the applied analytical method with the chemometric algorithm multivariate curve
89 resolution with alternating least-squares (MCR-ALS) [12] are demonstrated. Although the
90 combination of LC and second-order calibration has been reported in the literature [13], the
91 limits of the technique are still unknown in terms of the number of analytes that can be
92 quantified in highly interfering media. To the best of our knowledge, this is the first time that
93 eleven sex hormones are evaluated in challenging media using a GAC method, and second-

94 order calibration is applied to both high-performance liquid chromatography (HPLC)-DAD
95 and HPLC-FLD matrices measured for a single chromatographic run.
96
97 **2. Materials and Methods**
98
99 *2.1. Reagents and solutions*
100
101 AE, DES, E2, EE2, E3, E1, HEX, LEV, MEST, NOR, and PROG were purchased
102 from Sigma-Aldrich (Milwaukee, WI, USA). Methanol and acetonitrile were obtained from
103 Merck (Darmstadt, Germany). Water was purified using a MilliQ system (Millipore, Bedford,
104 USA). Solvents were filtered through 0.22 µm nylon filters.
105 Stock solutions of all analytes of about 2000 µg mL^{-1} were prepared in methanol.
106 From these solutions, more diluted methanol solutions (around 100 µg mL^{-1}) were obtained.
107 Working solutions were prepared immediately before their use by taking appropriate aliquots
108 of diluted methanol solutions, drying the solvent under a nitrogen stream and adding
109 acetonitrile and water (50:50 v/v) to the desired concentrations.
110
111 *2.2. Apparatus*
112
113 Chromatographic measurements were carried out on an HP 1200 liquid chromatograph
114 (Agilent Technologies, Waldbronn, Germany) equipped with degasser, quaternary pump, a
115 manual injector fitted with a 20 µL loop, a DAD, an FLD, and the HP ChemStation software
116 package for instrument control, data acquisition and data analysis. HPLC separations were
117 performed on a Poroshell 120 EC (4.6×100 mm, 2.7 µm particle size) column (Agilent
118 Technologies, Santa Clara, CS, USA).

119

120 *2.3. HPLC procedure*

121

122 Data matrices were collected every 1.8 s using wavelengths from 200 to 330 nm in
123 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,
124 setting the excitation wavelength at 275 nm and the slit widths at 1 nm. HPLC-DAD matrices
125 of size 580×131 and HPLC-FLD matrices of size 162×56 (time and spectral data points
126 respectively) were saved in ASCII format, and transferred to a PC for subsequent
127 manipulation. The mobile phase used was a 50:50 (v/v) mixture of water and acetonitrile,
128 delivered at a flow rate of 1.0 mL min^{-1} with a chromatographic system operating under
129 isocratic mode.

130

131 *2.4. Calibration and validation samples*

132

133 A calibration set of ten samples containing E3, E2, EE2, HEX and MEST in the range
134 0–50 ng mL^{-1} and the remaining compounds in the range 0–100 ng mL^{-1} was prepared (Table
135 S1 of Supplementary data). These concentrations were selected considering the low levels of
136 sex hormones usually found in natural samples (see below) and no efforts were made to
137 establish the upper concentration of the linear range. Eight samples of the set corresponded to
138 the concentrations provided by a semi-factorial design for four overlapped analytes (E1, DES,
139 AE and HEX) and equally spaced concentrations for those analytes with resolved bands. The
140 remaining calibration samples were a blank solution (with no addition of any of the eleven
141 analytes) and a mixture of all studied analytes at intermediate concentrations (e.g. ~ 25 and 50
142 ng mL^{-1}). A validation set of ten samples was additionally prepared, containing the analytes

143 in different concentrations than those used for calibration. Specific concentrations were taken
144 as random numbers generated within the calibration domain.
145
146 *2.5. Water samples*
147
148 Three different water samples (mineral, underground and river) were analyzed.
149 Underground (Funes City) and river water (Paraná River) samples were collected in amber
150 glass bottles, previously cleaned with methanol and Milli-Q water, and stored at 4 ºC after
151 sampling. Mineral water (Mendoza) was evaluated as purchased, while underground and river
152 samples were filtered with filter paper before their use.
153 Because none of the real samples contained the investigated compounds at larger
154 levels than the attained detection limits, a recovery study was performed spiking all water
155 samples with standard solutions of the analytes. For estrogens (except E1 and DES), the
156 ranges were 10–20 ng L^{-1} (low), 25–35 ng L^{-1} (medium) and 40–52 ng L^{-1} (high), whereas
157 for the remaining analytes they were 19–32 ng L^{-1} (low), 46–65 ng L^{-1} (medium) and 81–99
158 ng L^{-1} (high). The solid-phase extraction (SPE) procedure was carried out using SPE disks
159 Empore Octadecyl C18 (Supelco, Bellefonte, PA, USA). The membrane was conditioned
160 with 1 mL of methanol and then the extraction of 100 mL of the sample was carried out in
161 approximately 10 min per sample. This flow rate is in the optimum range for maximum
162 breakthrough volume [14]. The retained compounds were eluted with 0.5 mL methanol, and
163 this solvent was evaporated under a nitrogen stream. Then, the residue was reconstituted with
164 0.200 mL of mobile phase, filtered by a nylon filter before injection and finally subjected to
165 the same chromatographic analysis as the calibration samples. The preconcentration factor
166 was 1:500.
167

168 *2.6. Sediment samples*

169

170 Sediment samples from a water treatment plant (Rosario, Argentina), Paraná river and
171 Carcarañá river were collected in glass bottles, previously cleaned with methanol and Milli-Q
172 water. Since these samples did not contain detectable levels of the evaluated compounds, they
173 were spiked with standard methanol solutions in order to obtain concentration levels the range
174 2.5–24.3 ng g^{-1}. The fortified samples were then frozen and lyophilized in a Liotop L101
175 Liobras dryer (San Carlos, Brazil). Finally, they were ground using a mortar and stored at
176 –15 ºC until analysis. For the extraction procedure, 2.00 g of lyophilized sediment were
177 placed into a 25 mL beaker and treated with 5 mL of methanol. The mixture was ultrasonic
178 extracted for thirty minutes at room temperature and then was centrifuged at 10,000 *g* for ten
179 minutes. A portion of the supernatant was placed in a 100 mL volumetric flask, dried under a
180 gentle nitrogen stream and reconstituted with water to the mark.

181 The resulting solution was subjected to the same SPE procedure used for the water
182 samples with a preconcentration factor of 1:500.

183

184 *2.7. MCR-ALS algorithm and software*

185

186 The MCR-ALS theory is well documented in the literature [12] and only a brief
187 description is included in the Supporting Information. The data were handled using the
188 MATLAB computer environment [15]. The calculations involving MCR-ALS were
189 performed using MVC2, a new version of the already reported MATLAB graphical interface
190 toolbox [16], freely available on the Internet [17].

191

192 **3. Results and discussion**

193

194 *3.1. Preliminary considerations*

195

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

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

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

213 The resolution for some chromatographic bands of the DAD system is only partial
214 (Fig. 2). The picture is even more critical when the test sample is no longer a synthetic one
215 prepared in mobile phase, but a real sample, usually consisting of a significantly more
216 complex matrix. This latter situation affects both the DAD and FLD systems through severe

217 band overlapping. Therefore, the use of multivariate calibration through the processing of
218 HPLC-spectral second-order data is entirely justified.
219 Prior to constructing the experimental matrices, the characteristics of this type of data
220 must be considered. In chromatographic analysis, it is very common to observe the lack of
221 repeatability in the retention time and band shape of an analyte between successive runs. As a
222 result, the three-dimensional array formed with the chromatographic-spectral matrices
223 obtained loses the property of trilinearity [18]. Although this fact represents a serious obstacle
224 for algorithms which demand the trilinearity of the data [18], algorithms such as MCR-ALS
225 do not require this condition. They represent a valuable tool for the processing of this type of
226 data, for example by performing matrix augmentation in the temporal direction [12].
227 However, in the system under study, an additional problem must be taken into account: some
228 analytes exhibit very similar absorbance and fluorescence spectra (Fig. 3). In this situation, if
229 the full DAD and FLD chromatograms are processed, unsuitable results are obtained because
230 the mathematical pseudorank is smaller than the chemical rank [18]. To overcome this
231 inconvenience, MCR-ALS was applied with matrix augmentation in the temporal direction in
232 various selected time ranges, ensuring that each partial chromatographic region includes
233 analytes with different spectral profiles (Table 1).
234
235 *3.2. Analysis of calibration and validation sets*
236
237 MCR-ALS data processing comprised the building of augmented matrices in the
238 elution time direction containing, for each time region, a validation sample data and the
239 calibration data matrices. The number of components in each augmented matrix was
240 estimated by principal component analysis, and justified taking into account the presence of
241 the corresponding analytes and background signals. Non-negativity restrictions were applied

242 in both modes; unimodality restriction was applied in the elution time mode to the signals
243 corresponding to the analytes. The selected ALS convergence criterion was 0.01% (relative
244 change in fit for successive iterations), and in validation samples convergence was achieved
245 in less than 20 iterations. The residual fits for the DAD were lower than 0.04 mAU (milli
246 absorbance units), while those corresponding to FLD were about 0.01 UF (arbitrary units of
247 fluorescence), which is ca. 1% with respect to the maximum intensity measured. After
248 convergence of the ALS optimization for each sample, the constituents were identified and
249 quantification was carried out with the aid of the corresponding pseudo-univariate calibration
250 curves. Table 2 shows the parameters obtained for the latter regression curves corresponding
251 to a typical validation sample. The concentration prediction of each analyte proceeded by
252 interpolation into the corresponding pseudo-univariate score-concentration calibration plot.

253 Fig. 4 displays the good recovery results in validation samples in addition to the
254 elliptical joint confidence region (EJCR) [19] test for the slope and intercept of the plot
255 corresponding to each analyte. Because all ellipses include the theoretically expected values
256 of (1,0) for slope and intercept, respectively, the accuracy of the applied methodology for
257 these compounds in validation samples can be claimed. The statistical results corresponding
258 to validation samples are completed with the parameters shown in Table S2 of the
259 Supplementary data.

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

265

266 *3.3. Analysis of real samples*

267

268 The resolution of the samples selected as examples of environmental matrices for
269 evaluating the proposed methodology represents a real analytical challenge (Fig. 5). However,
270 MCR-ALS achieves the so-called “second-order advantage”, which avoids the major obstacle
271 of traditional zeroth-order calibration methods applied to complex mixtures: the requirement
272 of interference removal before the quantitative analytical method is applied [20].

273 MCR-ALS data processing was similar to that for validation samples, but in addition
274 to non-negativity in both modes and unimodality in the time mode restrictions, the
275 correspondence restriction was applied to most samples, which fixes the sequence and the
276 presence or absence of components in specific matrices [12]. In real samples, with an
277 unknown number of constituents, the number of components was estimated as in validation
278 (see above) and varied between 6 and 8, depending on the sample and analyzed time region.

279 The number of ALS iterations in these complex samples was less than 30 in most
280 cases, with residual fits in the order of the expected instrumental noise associated with each
281 detector. As in validation samples, after convergence was achieved, quantification was carried
282 out with the aid of the corresponding pseudo-univariate calibration curves.

283

284 *3.4. Water samples*

285

286 Concentrations of estrogens and progestagens in surface and wastewaters are normally
287 are lower than 20 ng L^{-1} [4,5,21,22]. However, larger amounts (e.g. E1, 51–3240 ng L^{-1}; E2,
288 451 ng L^{-1}; EE2, 178–410 ng L^{-1}; DES, 122 ng L^{-1}; NOR, 26–224 ng L^{-1}; AE, 10500 ng L^{-1};
289 PROG, 3470 ng L^{-1}) [6,21,23] can be sporadically found. Androgenic substances such as AE
290 are sometimes identified in rivers associated with paper mill effluents, and concentrations in
291 the range about 30–170 ng L^{-1} have been reported [24,25]. Water samples were spiked with

292 all analytes, combining random values from the corresponding concentration ranges and, after
293 a simple pre-concentration with a C18 membrane, each sample was processed as the
294 validation ones. Concentrations at sub-part per trillion could be measured with a larger pre-
295 concentration step (e.g. 1:2500) [11].

296 It is necessary to make a distinction between the presently proposed strategy, that only
297 needs to remove suspended particles in some natural waters from more strict extraction and/or
298 clean-up protocols usually employed in chromatographic analysis coupled to MS or tandem
299 MS for the determination of sex hormones in natural waters [5,7,26–30]. In our case, because
300 of the second-order advantage, soluble sample constituents injected in the chromatographic
301 column along with the analytes do not interfere in the analysis, as is demonstrated with the
302 successful MCR-ALS predictions (Table 3).

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

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

314 Table 3 also shows the statistical results for the analyzed samples. The relative errors
315 of prediction are very acceptable (smaller than 10 %) taking into account the complexity of
316 the studied samples. Limits of detection (LODs) were estimated based on rigorous IUPAC's

317 recommendations, which take into account type I and II errors (false positive and false
318 negative errors, respectively) and the error propagation from both the slope and the intercept
319 of the pseudo-univariate MCR-ALS calibration curve [31]:

320 $$\text{LOD} = 3.3(\text{SEN}^{-2}\ \sigma_x^2 + h_0\text{SEN}^{-2}\ \sigma_x^2 + h_0\sigma_{ycal}^2)^{1/2} \quad (1)$$

321 where the factor 3.3 is the sum of *t*-coefficients accounting for type I and II errors at 95%
322 confidence level, h_0 is the sample leverage at zero analyte concentration, σ_x^2 is the variance in
323 the instrumental signal, σ_{ycal}^2 is the variance in calibration concentrations, and SEN is the
324 component sensitivity [32]. LODs for the analytes determined by DAD, with an average value
325 of 17 ng L^{-1}, approximately double the LOD values for the analytes quantified by FLD (mean
326 LOD = 9 ng L^{-1}). This fact is ascribed to the different detector sensitivities. As expected, the
327 presence of a significant amount of interferents in a sample, such as a river one, produces a
328 deleterious effect in the calculated LODs.

329

330 *3.5. Sediment samples*

331

332 Concentrations of estrogens, progestagens and androgens in river sediments are in the
333 range of a few ng g^{-1} [4,5,24], and analytes were assayed at these levels.

334 The good recoveries and statistical values obtained (Table 4) are indicative of the
335 validity of the method and the effectiveness of the SPE procedure that enables the
336 quantification at very low analyte levels. As in the case of water samples, the results passed
337 the EJCR test (Fig. 6), demonstrating the accuracy of the employed methodology and how the
338 second-order calibration models the interferences naturally present in the studied complex
339 samples. Regarding this latter issue, it is also remarkable how the amount of organic solvents
340 was decreased using the proposed strategy, in comparison with that currently employed in
341 sample pre-treatments for the analysis of the studied hormones in sediments [5–7,27,33,34].

342

343 ## 4. Conclusions

344

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

354

355 ## Acknowledgments

356

357 The authors are grateful to the Universidad Nacional de Rosario, Consejo Nacional de
358 Investigaciones Científicas y Técnicas (CONICET, Project PIP 0163), and Agencia Nacional
359 de Promoción Científica y Tecnológica (PICT 2013-0136) for financially supporting this
360 work. R.L.P thanks CONICET for a doctoral fellowship.

361

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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

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]	
	DAD									
	VS	WS[e]	VS	WS[e]	VS	WS[e]	VS	WS[e]	VS	WS[e]
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.

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 ref. 31) 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).

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(0.4)	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 ref. 31) 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).

FIGURE CAPTIONS

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

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.

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).

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.

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).

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.

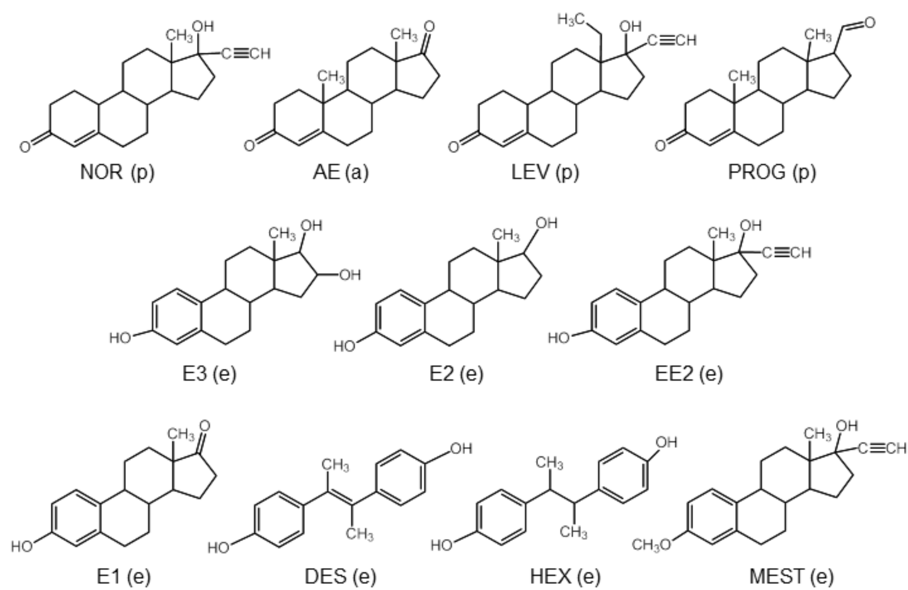

Figure 1

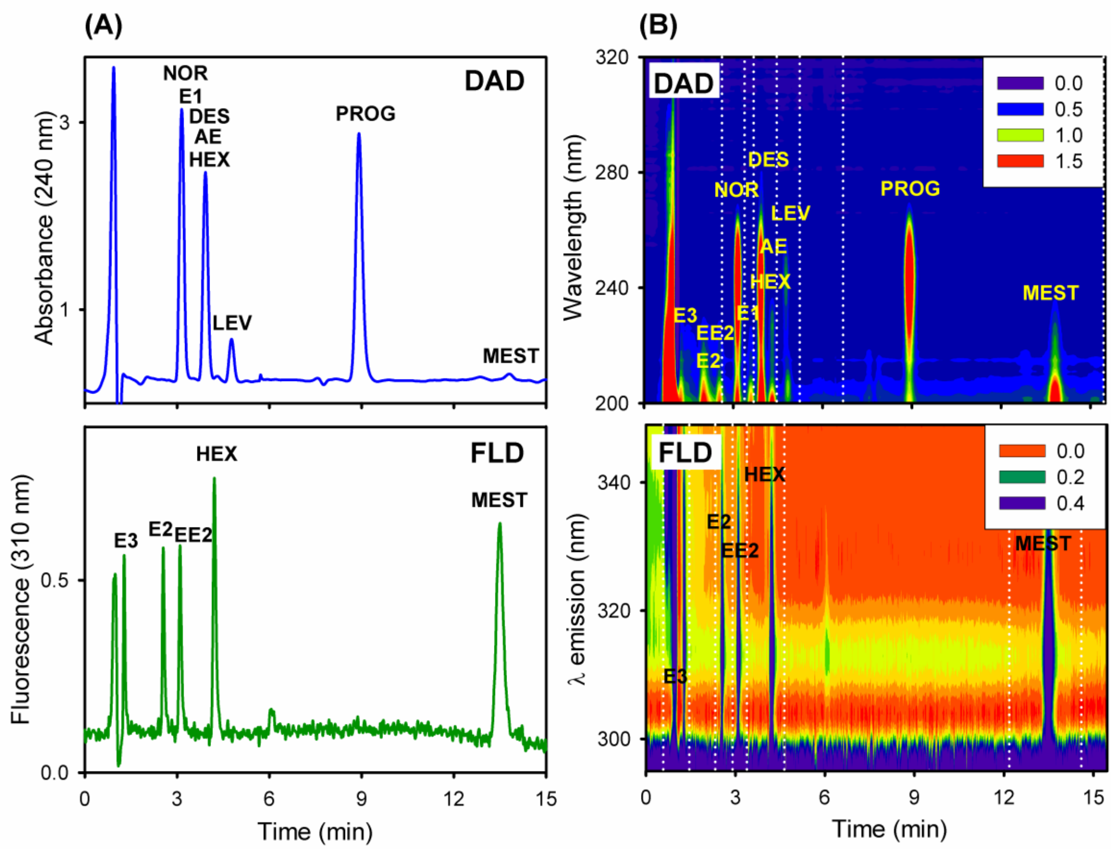

Figure 2

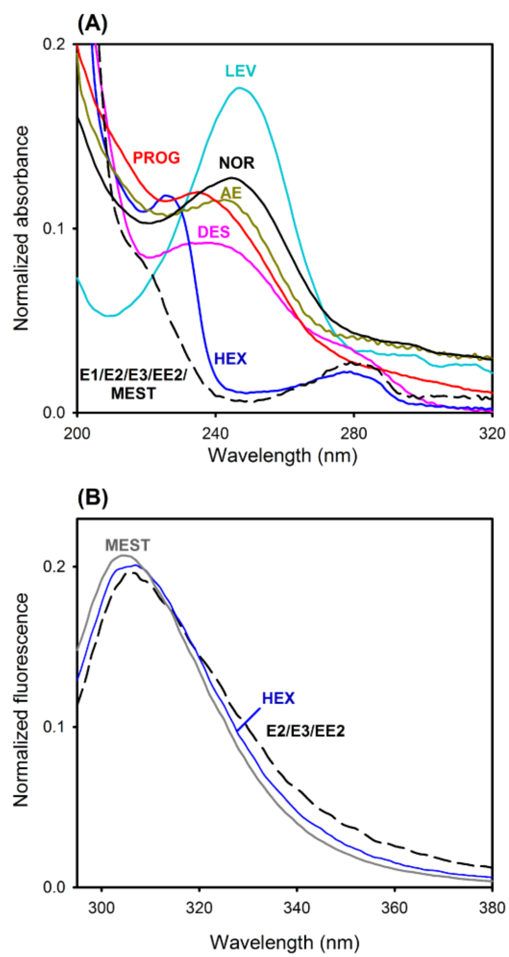

Figure 3

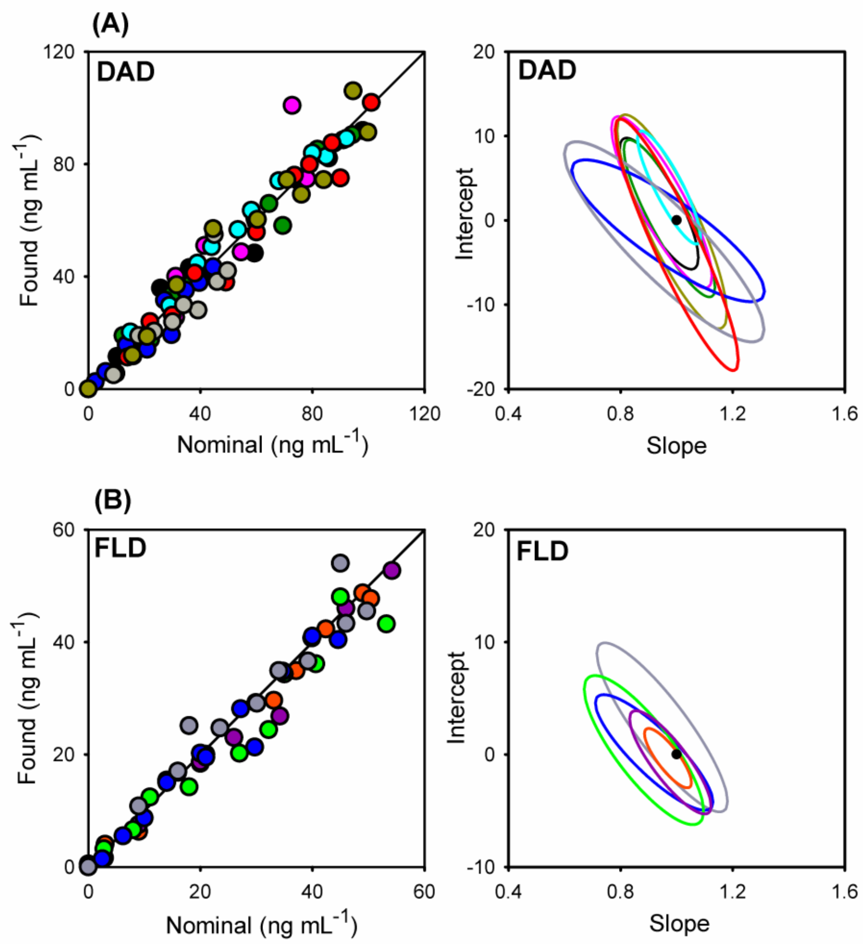

Figure 4

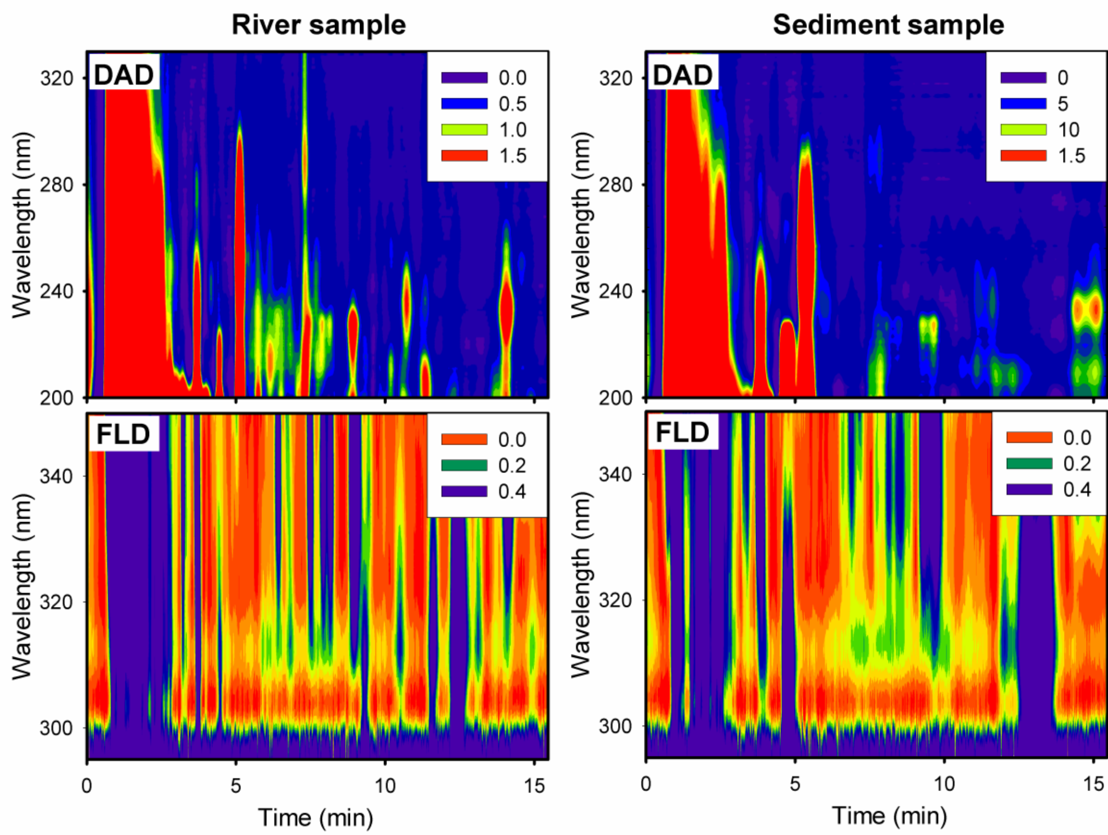

Figure 5

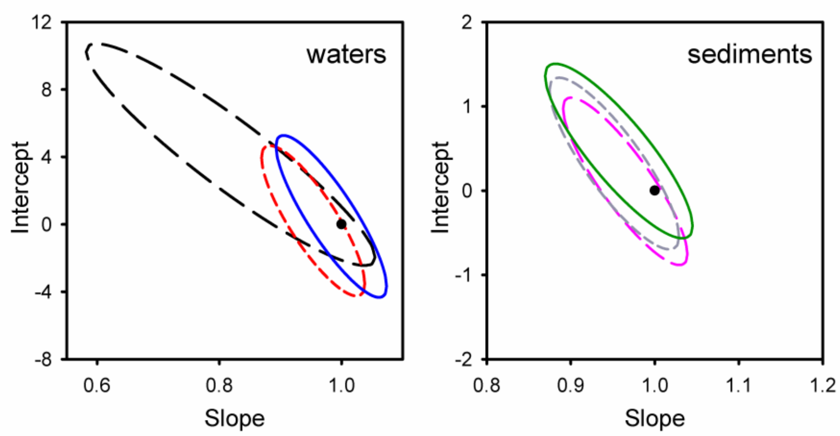

Figure 6