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1 **Development of a liquid chromatography - tandem mass spectrometry procedure for**
2 **determination of endocrine disrupting compounds in fish from Mediterranean rivers**

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11

12 **Abstract:** A new, sensitive and rapid method based on QuEChERS (Quick, Easy, Cheap,
13 Effective, Rugged and Safe) approach followed by ultra high performance liquid
14 chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) was developed
15 for the determination of nineteen endocrine disruptors (EDCs) and related compounds
16 belonging to different classes in various fish species. Matrix effect on the analytical
17 performance was evaluated, and thus, internal sample calibration was chosen as the most
18 appropriate approach when analyzing such complex matrices as biota. The procedure
19 provided adequate recoveries in the range from 40% to 103% for most of the compounds, low
20 method detection limits (MDLs) in the range from 0.002 to 3.09 ng/g for fish homogenates
21 and high accuracy <20%. The developed method was applied for the analysis of target
22 compounds in homogenates of different fish species from four impacted Mediterranean rivers:
23 Ebro, Llobregat, Júcar and Guadalquivir. Eleven out of the nineteen target EDCs were found
24 at least once in fish homogenates. Llobregat was identified as the most polluted river, where
25 high concentrations were measured in fish homogenates especially for bisphenol A
26 (223.91±11.51 ng/g). Tris (2-butoxyethyl) phosphate (TBEP), caffeine, and methyl and benzyl
27 paraben were found in fish from the four river basins.

28

29 **Key words:** Endocrine disrupting compounds, hormones, fish, QuEChERS, UHPLC-MS/MS

30

31



31 1. Introduction

32 Endocrine disrupting compounds (EDCs) are considered “emerging” or “new”
33 unregulated contaminants and have received particular attention in recent years since they can
34 affect the environment and living organisms. EDCs include natural and synthetic compounds
35 that have the ability to mimic the function of the endogenous compounds or affect the
36 reproductive action of the endocrine system in animals and humans [1,2]. EDCs have proved
37 to cause many negative effects such as behavioral disorders [3,4], infertility [5], birth
38 malformations [6] or feminization of male fish [7,8]. The mechanisms of these pathologies are
39 very complex and dependant on enzymatic activities, which are responsible for balance of
40 androgens and estrogens, disrupted by EDCs [1]. Endocrine disrupting compounds can be
41 divided into two groups: natural steroid hormones formed from cholesterol [9,10] and
42 xenobiotics which include synthetic steroid hormones (e.g. 17- α -ethinylestradiol) and man-
43 made chemicals (e.g. surfactants, flame retardants, pesticides and pharmaceuticals) [11].
44 Currently, there are many research reports that confirm the presence of many types of EDCs
45 in the environment, mostly including surface water [12,13], wastewater [10], sediment
46 [12,14,15], sewage sludge [16,17], in biological samples such as urine [18] or serum [19] and
47 even in drinking water [20].

48 Major source of EDCs in aquatic environment are the effluents from wastewater
49 treatment plants (WWTPs), since conventional WWTPs processes based on activated sludge
50 are not able to remove EDCs completely [10]. Therefore, aquatic organisms in rivers impacted
51 by WWTP effluents are continuously exposed to low doses of EDCs. Research on aquatic
52 organisms are of special interest since some of the EDCs such as bisphenol A (BPA) and
53 triclosan are prone to bioaccumulate [21,22] due to the high octanol-water partition
54 coefficients [23] of 4.04 and 4.98, respectively. The study of the presence, impact and effects
55 of these contaminants on wild fish in particular is very important since they play an important
56 role in aquatic food chains and are exposed to the pollutants present in sediments, overlying
57 water and in their food (algae, invertebrates and other fish). To properly predict the impact of
58 EDCs on hormonal system it is necessary to simultaneously detect and quantify endogenous
59 hormones and EDCs [24]. However, the determination of EDCs in fish can be troublesome
60 due to matrix complexity (fish may contain a high level of lipids), and therefore, demand a
61 highly thorough sample pre-treatment.

62 There are several publications reporting the determination of EDCs in fish sample
63 obtained by homogenization of whole fish individual [25]; or tissues [22,26]; however, they
64 mostly include only a few compounds or at most one group of compounds. There is only a
65 few papers published so far which allow for the determination of EDCs from different groups
66 (i.e. hormones, alkylphenols, BPA) in biota [15,22,27]. In addition, the vast majority of the
67 analytical procedures are based on a time- and/or solvent- consuming techniques such as
68 accelerated solvent extraction (ASE) [21,25,28-30], high speed solvent extraction [26,31] or
69 sonication [27,32]. Furthermore, extracts from such complex fish matrices also require a
70 clean-up method since high lipid content may interfere chromatographic separation and
71 analysis of target analytes. The most often applied purification step include solid phase
72 extraction (SPE) using Florisil adsorbent [27,33], C18 cartridge [21] or gel permeation
73 chromatography (GPC) [33]. Among the more recent sample preparation approaches
74 QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) has been successfully applied
75 for mammal tissues [34], for the determination of hormones in shrimp [35] and EDCs in rat
76 testis [1]. The major advantages of QuEChERS sample preparation are low usage of solvents
77 (following low costs), simplicity, extraction speed, high sample throughput and possibility to
78 obtain high recoveries for a wide spectrum of compounds. However, there are no analytical
79 methods based on QuEChERS which allow for the determination of a wide range of EDCs in
80 fish samples.

81 On the other hand, analysis of hormones and EDCs are mostly performed by two
82 techniques, either gas chromatography coupled with (tandem) mass spectrometry [33,36,37]
83 or liquid chromatography coupled with tandem mass spectrometry [21,31]. However, using
84 gas chromatography requires additional step as derivatization or hydrolysis, which may cause
85 losing information about hormone conjugates (e.g. sulfate and glucuronide) [1,38].

86 In light of the lack of multi-residue analytical methods for the determination of several
87 EDCs in very complex samples and the interest of analyzing them in aquatic biota, the aim of
88 the present work was to develop a multi-residue procedure for the simultaneous determination
89 of 19 main concerned EDCs present at ultra-trace levels in fish homogenates. Different
90 sample preparation procedures were tested; and the most appropriate methodology was based
91 on QuEChERS extraction followed by liquid chromatography coupled with tandem mass
92 spectrometry (LC-MS/MS) analysis. This paper describes the comparison of different sample
93 preparation techniques and also the optimization and validation of the analytical method of
94 choice. According to our knowledge, this is the simplest and most rapid procedure that has

95 been successfully applied to fish samples allowing the simultaneous determination of 19
96 multi-class EDCs (19 compounds from different classes: triazoles, stimulants, hormones,
97 flame retardants, plasticizers, antibacterials, preservatives). This methodology is competitive
98 in terms of number of EDCs determined in one analytical cycle, sensitivity, rapidity (is faster
99 than the other methods published) and efficiency. The QuEChERS-LC-MS/MS methodology
100 is applied for the first time for the simultaneous determination of trace levels of 19 EDCs in
101 fish and provides good recoveries and low limits of quantification and was further applied to
102 determine the presence of the target compounds in wild fish collected during a monitoring
103 campaign in four different river basins in Mediterranean area.

104

105 **2. Materials and methods**

106 *2.1. Standards and reagents*

107 High purity standards for target compounds summarized in Table 1 were purchased
108 from Sigma-Aldrich. Isotopically labeled compounds, used as internal standards, estrone-d4,
109 17 β -estradiol-d2, 17 α -ethinylestradiol-d4, BPA-d4, methylparaben-d4, triclosan-d3, 1H-
110 benzotriazole-d4 and caffeine-d3 were purchased from CDN isotopes. Progesterone-d8 was
111 from Cambridge Isotope Laboratories. Trisphenylphosphate-d15 was obtained from Sigma-
112 Aldrich.

113 Individual stock solutions and isotopically labeled internal standards were prepared in
114 methanol at a concentration of 1000 mg/L and stored at -20°C. Stock mixtures of 20 mg/L
115 were prepared in methanol and stored in the same conditions. Working standard solutions of
116 EDCs and internal standards (ISs) (1 mg/L), as well as standard solutions for calibration curve
117 were diluted with methanol/water (1:1, v/v) before each analytical run.

118

119 *2.2. Sample collection and preservation*

120 Fish samples were collected during the summer 2010 from four Mediterranean rivers
121 (Ebro, Llobregat, Júcar and Guadalquivir) in Spain. Five points were sampled along each river
122 (Fig 1). Fish individuals belonged to 11 different species: *Barbus graellsii*, *Micropterus*
123 *salmoides*, *Cyprinus carpio*, *Salmo trutta*, *Silurus glanis*, *Anguilla anguilla*, *Lepomis*
124 *gibbosus*, *Gobio gobio*, *Luciobarbus sclateri*, *Aburnus alburnus*, and *Pseudochondrostoma*
125 *willkommii*. Whole individuals (n=3) from each species were homogenized by a meat grinder,
126 freeze-dried and stored at -20°C until analysis. Lipid content was measured for fish
127 homogenate for several species following the method developed by Spiric *et al.* [39].

128

129 2.3. Optimization of sample preparation step

130 Different sample preparation methods were tested in order to select and optimize the
131 most suitable one for determination of EDCs in fish samples. Three extraction methods were
132 initially selected for comparison on the basis of their applicability to biota samples and
133 rapidity. 1g of freeze-dried fish homogenates of *Barbus graellsii* were first spiked at a final
134 concentration level of 20ng/g with a mixture of EDCs and subsequently subjected to the
135 different extraction procedures in order to obtain the best recovery results for the target
136 analytes.

137 The first extraction protocol was based on Huerta *et al.* method [40] and consisted on
138 pressurized liquid extraction (PLE) followed by gel permeation chromatography (GPC) clean-
139 up. PLE conditions included methanol extraction in 4 cycles of 5 min each at 50°C for 1 g
140 sample of fish homogenate. Final extracts were evaporated to dryness under a stream of
141 nitrogen, reconstituted in methanol and subjected to GPC purification step carried with an
142 Agilent 1260 Infinity high pressure liquid chromatography system with a diode array detector
143 (HPLC-DAD) using an Agilent EnviroPrep column (300x21.1 mm, 10 µm) coupled to a
144 PLgel guard column (50x7.5 mm). Mobile phase was DCM/MeOH (90:10 v/v) at flow rate of
145 5 mL/min in isocratic conditions and injection volume was 250 µL. Fractions containing
146 target compounds were collected between 13.5 to 26.5 min and subsequently evaporated to
147 dryness.

148 The second extraction method was PLE followed by Florisil clean-up similarly to
149 Gorga *et al.* [41] method. Analytes were extracted with a mixture of acetone/MeOH/H₂O
150 (1:2:1 v/v/v) in 4 cycles of 5 min each at 50°C using 1 g of a fish homogenate. Final extracts
151 were evaporated to dryness under a stream of nitrogen, reconstituted in 8 mL of ACN and
152 subjected to clean-up procedure. Purification was carried out with Florisil cartridges (Agilent
153 Technologies) which were conditioned with 5 mL portion of hexane and 5 mL of ACN. Later
154 acetonitrile extracts were passed through a sorbent, followed by 2 mL of ACN. All was
155 collected and evaporated to dryness.

156 The third approach based on QuEChERS (QuEChERS Kits, Agilent Technologies)
157 involved two steps, extraction with acetonitrile in aqueous conditions followed by the
158 application of specific salt (4g MgSO₄, 1 g NaCl) used for salting out of water from the
159 sample and to induce liquid-liquid partitioning; and purification with dispersive solid phase
160 extraction (dSPE) using sorbent mixture (900 mg MgSO₄, 150 mg PSA (primary and
161 secondary amine exchange material), 150 mg C18). Once QuEChERS was chosen as the



162 procedure which provided the best results for target compounds, the best conditions were
163 further optimized for fish homogenate. Three extraction salts: (I) 4g MgSO₄, 1 g NaCl; (II)
164 1.5 g sodium acetate, 6 g MgSO₄; (III) 4 g MgSO₄, 1 g NaCl, 1 g trisodium citrate dehydrate,
165 0.5 g disodium hydrogencitrate sesquihydrate, and four dSPE sorbents: (I) 900 mg MgSO₄,
166 150 mg PSA; (II) 900 mg MgSO₄, 150 mg PSA, 150 mg C18; (III) 400 mg PSA, 400 mg C18,
167 400 mg GCB (graphitized carbon black), 1200 mg MgSO₄; (IV) 150 mg PSA, 15 mg GCB,
168 900 mg MgSO₄, were tested in different combinations. After choosing the best extraction salt
169 – dSPE sorbent pair other parameters such as sample weight (0.5g, 1g, 1.5g) and a volume of
170 ACN added to reach different V_{ACN}/V_{water} ratios of 4:1, 2:1 and 4:3, were optimized. Also a
171 different approach, which includes application of hexane as a purification solvent instead of
172 typical dSPE sorbent, similar to the one proposed by Pouech *et al.* [1] was tested. For hexane
173 purification, a specific volume of hexane was added right after ACN portion leading to a
174 V_{ACN}/V_{hexane} ratio of 2:1, vortexed for 30 s and as follows in the overall procedure excluding
175 the dSPE step.

176 All extracts after drying were reconstituted in 0.5 mL of MeOH/H₂O (1:1, v/v) and
177 finally, 10 μ L of IS mixture was added and vortexed with the sample thoroughly before LC-
178 MS/MS analysis.

179 The final QuEChERS procedure for the extraction and purification of selected EDCs
180 in fish homogenates was the following: 0.5 g of homogenized and freeze-dried fish sample
181 was transferred to a 50-mL polypropylene centrifuge tube and vortex for 30 s. Then a ceramic
182 homogenizer and water was added and vortexed for 30 s. After vortexing for 1 min with
183 subsequent addition of ACN, an extraction salt was added directly to the tube and then the
184 mixture was immediately manually shaken for 1 min to avoid agglomeration of salts. Samples
185 were centrifuged at 11,000 rpm for 4 min. Then the ACN layer was transferred to the
186 polypropylene tube containing dSPE sorbents, vortexed for 1 min and centrifuged for 15 min
187 at 5,000 rpm. Later 5 mL of the extract was evaporated to dryness.

188

189 2.4. UHPLC-MS/MS analysis

190 LC analysis were performed on a Waters Acquity Ultra-Performance™ liquid
191 chromatography system equipped with two binary pumps systems (Milford, MA, USA), using
192 an Acquity BEH C18 column (50 mm \times 2.1 mm i.d., 1.7 μ m particle size) purchased from
193 Waters Corporation and applied for both ionization modes. The optimized separation
194 conditions were as follows: solvent (A) methanol and (B) water (pH 9, adjusted with
195 ammonia) at a flow rate of 0.4 mL/min. The gradient elution for positive ion mode (PI) was:

196 0-3 min, 30-100% A; 3-4.75 min, 100% A; 4.75-5.75 min return to initial conditions; 5.75-7
197 min, equilibration of the column and for negative ion (NI) mode: 0-4 min, 30-100% A; 4-5
198 min, 100% A; 5-6 min return to initial conditions; 6-7.5 min, equilibration of the column. The
199 column was maintained at 40°C in NI; the temperature was not controlled in PI. The sample
200 volume injected was 5 µL for both ion modes. Chromatogram of the separation of 19 EDCs
201 and related compounds are presented in Figure S1.

202 The UHPLC instrument was coupled with a 5500 QTRAP hybrid triple quadrupole-
203 linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an
204 electrospray interface. Compound dependent MS parameters (declustering potential (DP),
205 collision energy (CE) and collision cell exit potential (CXP)) as well as compound Selected
206 Reaction Monitoring (SRM) transitions were optimized by direct infusion of individual
207 standard solution of each analyte at 10 µg/L. A summary of these parameters is presented in
208 Table 2. All transitions were recorded in Scheduled MRM algorithm with 30s detection
209 window. Source dependent parameters were determined by Flow Injection Analysis (FIA) and
210 are as follows: curtain gas (CUR) - 30 V, nitrogen collision gas (CAD) - medium, source
211 temperature - 600°C, ion spray voltage - 5000 and 3000 V, ion spray gases GS1 - 60 V and
212 GS2 - 40 V for compounds analyzed under PI and NI, respectively. Instrument control data
213 acquisition and data analysis were carried out using Analyst software (Applied Biosystem).

214

215 3. Results and discussion

216 3.1. Optimization of sample preparation step for fish homogenates

217 Three preliminary sample pretreatment protocols based on PLE extraction with GPC
218 clean-up, PLE extraction with Florisil clean-up and QuEChERS were compared in term of
219 extraction efficiencies for the 19 endocrine disrupting compounds. Fish samples are
220 characterized by high lipid content, from 10-15 % (*Cyprinus carpio* and *Barbus graellsii*) to,
221 25 % (*Silurus glanis* - up to) [40], and therefore, in addition to the corresponding extraction
222 step, it was necessary to include a purification step through either GPC analysis or Florisil
223 sorbents. The extraction efficiencies for PLE with GPC clean-up was considered as an
224 inefficient method as only five out of the nineteen compounds had recoveries higher than 40%
225 (Fig. 2). In addition, the compounds, estrone-3-sulfate and triclosan were not recovered at all.
226 Results obtained for PLE with Florisil clean-up, allowed the extraction of most of the
227 compounds (Fig. 2); however, estrone metabolite was still not extracted from the matrix.
228 Although the extraction of compounds from solid matrices should be better in PLE, as it is
229 assisted by high temperature and pressure, it also co-extracts other matrix components, which

230 may not be sufficiently removed during the purification step leading to high matrix effects.
231 QuEChERS method, which includes micro-scale extraction and purification with dispersive
232 solid phase extraction (dSPE), was finally chosen as the most efficient method: QuEChERS
233 approach allowed the simultaneous extraction of all target compounds and provided
234 satisfactory recoveries (Fig. 2) surpassing 40% for the most relevant analytes and low values
235 of relative standard deviation (RSD%). Application of QuEChERS may cause co-extraction of
236 non-target compounds as well and therefore, it was crucial to perform further optimization
237 tests with different extraction salts and sorbents used for dSPE. Each extraction salt (3
238 different in total) was tested with the 4 purification sorbents selected, giving in overall twelve
239 pairs. The best results were obtained for the combination of the extraction salt composed with
240 1.5 g sodium acetate, 6 g MgSO_4 , thus only combinations based on this salt with different
241 sorbents are presented in the Fig. 3. As it can be seen, the second dSPE sorbent (900 mg
242 MgSO_4 , 150 mg PSA, 150 mg C18) gave the most satisfactory results since this sorbents
243 mixture is dedicated to samples with high lipid content [Technologies, 2011 #64]. The
244 recoveries exceed 50% for most of the target compounds and low RSD% values <18% were
245 achieved. Furthermore, only this particular dSPE sorbent provided much higher extraction
246 efficiency for such relevant compounds as BPA, triclosan, estrone and its metabolite estrone-
247 3-sulfate. The purification with hexane instead of dSPE as suggested by Pouech et al. [1] was
248 tested as well; however, high matrix effects were observed leading to the final optimal
249 recovery for only five out of nineteen EDCs.

250 Regarding the acetonitrile/water ratio of the solvent mixture used during first step of
251 QuEChERS extraction, Fig. 4 presents the recoveries for the analyzed EDCs obtained for the
252 three solvent mixtures applied: $V_{\text{ACN}}/V_{\text{water}}$ of 4:1, 2:1 and 4:3. Even though, the best results
253 for most of the compounds were obtained for the mixture with the highest content of water,
254 $V_{\text{ACN}}/V_{\text{water}}$ ratio of 4:3, significant low recoveries for compounds of high importance such as
255 BPA, 17β -estradiol and 17α -ethinyloestradiol were obtained. On the contrary, the extraction
256 mixture with the highest ACN content ($V_{\text{ACN}}/V_{\text{water}}$ 4:1) provided overall lower extraction
257 efficiencies for most of the compounds, and thus the final $V_{\text{ACN}}/V_{\text{water}}$ ratio was set at 2:1.

258 Several other parameters as sample amount (0.5 g, 1 g, 1.5 g) and extraction time after
259 salt addition (30 s, 60 s) were also optimized. The final sample size was set to 0.5 g since
260 higher recoveries and lower RSD% values were obtained for most of the target compounds
261 (data not shown). Additionally, the amount of co-extracted component was reduced, thus
262 limiting the influence of the matrix. The extraction time was set at 60 s, since the longer
263 extraction time significantly increased the recoveries (data not shown).

264

265 3.2. Matrix effect

266 A significant drawback in the MS analysis performed with electrospray (ESI) as
267 ionization technique is the appearance of matrix effect, especially when analyzing complex
268 matrices such as fish. This occurs due to the high sensitivity of ESI source to different
269 components present in the matrix, which can lead to signal suppression or enhancement,
270 thereby leading to false quantitative results. A thorough evaluation of matrix effect (ME%) for
271 fish homogenate was thus performed by comparing the peak area of the target compound in
272 fish extract spiked at 10 ng/g (after previous subtraction of the peak area of the analyte present
273 in the extract) with the peak area of the analyte in the solvent (MeOH/H₂O 1:1 v/v) at the
274 same concentration level. The percentage of matrix effect was then calculated according to the
275 equation: $ME\% = (A_{\text{matrix}}/A_{\text{solvent}} - 1) \times 100$ [28]. Calculations were performed in triplicate for
276 three fish species (*Cyprinus carpio*, *Barbus graellsii* and *Silurus glanis*) and the values
277 obtained are presented in Fig. 5. The results for the different fish species indicate that ion
278 suppression was observed for all EDCs. The lowest ME% was observed for estrone-3-sulfate
279 (-11.6% for *Silurus glanis*); however, for the other compounds matrix effects were high up to
280 98% for estrone, 17 β -estradiol and triclosan for *Barbus graellsii*.

281 In order to overcome ion suppression different approaches that should include the
282 variability of the matrices can be undertaken, such as selective and efficient purification of the
283 sample prior to analysis. However, such approach is not always appropriate and may lead to
284 analyte loss or increase of analysis time. Different, reliable and effective strategies described
285 in the literature are based on appropriate calibration methods, such as standard addition,
286 internal standard with isotopically labeled standards or matrix-matched calibration [42].
287 Although the best choice is the application of standard addition, it is a very time-consuming
288 approach due to the high amount number of different samples to process. Internal standard
289 calibration, on the other hand, is based on the addition to the sample extract of isotopically
290 labeled compounds that are structurally similar to the target analytes. This allows the
291 correction of the matrix effect since internal standard undergoes the same interferences as the
292 analytes. However, only ten isotopic analogues out of the nineteen target compounds were
293 available, and thus, this method did not seem the most appropriate for an accurate
294 determination of the whole set of target compounds. A good alternative is the application of
295 matrix-matched calibration, but it requires matrices (similar to the one analyzed) free from the
296 target compounds, which was not possible in this case. Therefore, a different strategy
297 previously applied by Stüber *et al.* [43], called internal sample calibration, was adopted. This

298 approach combines advantages of both, matrix-matched and internal standard calibration,
299 enabling the correction of the matrix effects for all the target compounds. For this purpose,
300 calibration curves were prepared in fish extracts for each fish species, with addition of
301 available isotopically labeled compounds and were presented as a dependence of the ratio of a
302 peak area of an analyte and a peak area of an internal standard to an analyte concentration.
303 The internal sample calibration prepared in the matrix which is consistent or similar to the fish
304 species analyzed was considered as the best approach for the determination of EDCs in biota
305 samples.

306

307 3.3. Method validation

308 The determined validation parameters were method detection limit (MDL), method
309 quantification limit (MQL) (Table 3), recovery (Table 4) accuracy and precision (Table 5).
310 Each parameter was determined for each of the three representative fish species (*Cyprinus*
311 *carpio*, *Barbus graellsii* and *Silurus glanis*). Moreover, since it was impossible to obtain a
312 blank matrix, the validation was performed using fish homogenates where some of the targets
313 EDCs were expected to be present at diverse concentrations. Therefore, to get a homogenous
314 representative fish homogenates, a mixture of 20 fish extracts was prepared separately for
315 each species. To determine the amount of present EDCs, a non-spiked extract was analyzed at
316 the same time than the rest of validation extracts. All spiked and non-spiked extracts were
317 obtained from the same matrix (e.g. fish species).

318 MDL and MQL were defined as the lowest analyte concentrations that can be detected
319 or quantified and determined for signal-to-noise ratios of 3 and 9, respectively. Both
320 parameters were determined in spiked samples (n=3) of the three matrices considered and are
321 summarized in Table 3. MDLs for the target compounds ranged from 0.002 to 3.09 ng/g and
322 were generally similar or lower comparing to the currently published procedures for some
323 target compounds [15,26,27,31].

324 Total recoveries were calculated for two spiking levels, 10 and 100 ng/g, which were
325 set as the lower and higher level of expected EDCs levels in fish samples based on literature.
326 Recoveries were determined for the final sample preparation method for fish homogenates
327 and were calculated by internal sample calibration. Results, summarized in Table 4, were
328 obtained for the three representative species *Cyprinus carpio*, *Barbus graellsii* and *Silurus*
329 *glanis*. Recoveries ranged from 40.1 ± 19.8 (benzylparaben) to 103.1 ± 3.7 (estrone-3-sulfate)
330 for *Barbus graellsii*; from 48.9 ± 9.1 (17 α -ethinylestradiol) to 113.2 ± 8.8 (propylparaben) for
331 *Cyprinus carpio*; from 34.2 ± 9.1 (1H-benzotriazole) to 90.5 ± 0.5 (triclosan) for *Silurus*

332 *glanis* (except for estrone-3-sulfate) for lower spiking level. Recoveries higher than 40% were
333 accepted [27,44] since the determination of EDCs in biota matrices is a challenging issue for
334 the following reasons i) EDCs belong to different classes, thus they differ in physic-chemical
335 properties (e.g. lipophilicity, log P, pKa); ii) biota samples contain a high amount of
336 interferences which have a significant influence at sample preparation step as well as LC-
337 MS/MS analysis (e.g. high lipid content and non-target compounds which are co-extracted).
338 However, recoveries for some compounds in case of specific species (e.g. estrone recoveries
339 for *Silurus glanis*) were lower for higher spiking level (100 ng/g). That phenomenon can be
340 explained by the decrease of extraction and purification efficiency when working at high
341 concentrations. This is due to the fact that the capacity of solvent during extraction (first step)
342 and sorbents in dSPE during purification (second step) is limited.

343 Accuracy and precision were calculated from six repeated injections of a spiked
344 extract at concentration level of 20 ng/g. Accuracy was expressed as the percentage value of
345 the bias between the theoretical and calculated concentrations, as described by Pouech *et al.*
346 [1]. As it can be seen in Table 5, the bias values were acceptable, lower than 20%. It can be
347 concluded that the bias values are higher than 10% in case of the compounds which internal
348 standard was not its isotopically labeled analogue. Precision was expressed as the percentage
349 value of the relative standard deviation of the measured concentration. RSDs values were
350 lower than 13% for the three fish species considered (Table 5).

351 The calibration curves for each analyte were based on internal sample calibration and
352 were generated for three different fish using linear regression analysis in the concentration
353 range 0.01 to 200 ng/g (when MDL of the compound was higher than 0.01 ng/g the lowest
354 concentration for the calibration curve was correspondingly higher). The response of each
355 compound was linear in the established concentration range, and all coefficients of
356 determination were greater than 0.99.

357

358 3.4. Application to real samples

359 The developed QuEChERS-UHPLC-MS/MS method was applied for the
360 determination of the target endocrine disrupting compounds in 50 samples corresponding to
361 12 different fish species from four Mediterranean rivers (Ebro, Llobregat, Júcar and
362 Guadalquivir). These rivers receive high pollution loads from anthropogenic activities, where
363 emerging pollutants such as EDCs are continuously released from WWTP (hormones and
364 personal care products and plastic derived products such as bisphenol A (BPA)), livestock



365 industry and textile industry (flame retardants), cosmetic and pharmaceutical industry
366 (parabens, antimicrobial, triazoles).

367 Eleven out of the nineteen target EDCs were found at least once in fish homogenates.
368 In general, detection frequency varied from 2% for estrone and tris(2-chloroethyl) phosphate
369 (TCEP) to 71% for tris(2-butoxyethyl) phosphate (TBEP) considering the four river basins.
370 Caffeine was detected also recurrently (48.8%) as well as methylparaben (46.3%). Hormones
371 were not found in any of the samples analyzed except for one sample in Ebro River, where
372 estrone was detected in a *Cyprinus carpio* sample at 1.99 ng/g. Similar concentration was
373 found for estrone and other hormones such as estradiol, estriol and ethinylestradiol in fish
374 from a contaminated region in Taiwan [45] and from supermarket in China [15]. BPA was
375 found in one sample in Guadalquivir river at 59.09±8.12, and at a maximum of 223.91 ng/g in
376 Llobregat River. To the author knowledge, this are the highest levels found for any of the
377 target compounds analyzed in this work, as well as the highest BPA concentration in wild, as
378 values reported so far ranged between 1 to 83 ng/g [15,27,45,46]. BPA, known to exhibit
379 estrogenic activity, can be associated to reproductive cancers, fertility problems and other
380 endocrine related endpoints [47], which raise a concern about its presence in fish. Triclosan,
381 which is a broadly used antibacterial compound, was also found in 15% of the samples
382 analyzed, being ubiquitous in Guadalquivir river where 80% of the samples analyzed
383 exhibited values between 1.98 to 17.41 ng/g. Triclosan was detected in different fish samples
384 in monitoring studies performed in Europe and Asia [26,31,48,49] and even 570 ng/g was
385 detected in fish samples from Manila Bay in Philippines [31]. The stimulant caffeine, on the
386 other hand, was found in more than 50% of the samples analyzed along the 4 rivers. Levels of
387 caffeine were between 0.56 to 21.40 ng/g, up to one order of magnitude higher than those
388 reported in USA by Wang et al. [50], which to the author's knowledge is the only study that
389 have reported caffeine bioaccumulation in fish samples. No previous study has reported the
390 presence of the tolytriazole, which was found at 1.25 ng/g in one sample of Ebro River and at
391 10.18 ng/g in another fish sample at Llobregat River. The most ubiquitous contaminants in
392 fish samples was the flame retardant TBEP, found in the 75% of all samples analyzed at
393 values up to 52.96 ng/g. This is a well known contaminant which was previously detected in
394 herring gull eggs in the concentration range 0.16-2.2 ng/g w.wt. [51] and in flathead grey
395 mullet at 11.6 ng/g l.w. [26]. Chen *et al.* [51] suggested that consistent detection of TBEP,
396 despite its low value of octanol/water partition coefficient, may indicate its potential to
397 bioaccumulate.



398 Finally, levels for paraben preservatives found in fish homogenates ranged from
399 0.19 ± 0.04 ng/g for propylparaben, to 84.69 ± 6.58 ng/g for methylparaben (Júcar river); but
400 still below the extremely high concentrations found by Kim et al. [26] and Ramaswamy et al.
401 [31] in fish muscle tissues taken in Manila Bay (Philippines) for methylparaben, ethylparaben
402 and propylparaben: up to 3450, 183 and 1140 ng/g, respectively. Benzylparaben, which was
403 not studied in cited articles, has been detected for the first time in the present study, in
404 Mediterranean Rivers. Even though, levels found were below ng/g range, it was present in
405 22% of the sample in all rivers considered, and can thus be considered one of the most
406 ubiquitous compounds of the study, after TBEP, caffeine and methylparaben.

407 Jucar river samples were comparatively less polluted than the rest of the fish samples.
408 EDCs contaminants were detected in the 9 different fish species sampled but at relatively
409 lower concentrations than in other rivers. The highest values for methylparaben,
410 propylparaben caffeine and TBEP were found in a *Salmo trutta* sample at the sampling point
411 JUC2, which corresponds to a river site impacted by the effluents of urban wastewater
412 treatment plant of Cuenca (57032 inhabitants). The rest of sampling sites are not as polluted
413 as those from JUC2 and low levels of EDCs in water and sediments have been reported in
414 accordance [41,52] and as it is shown in table 6B.

415 Guadalquivir cannot be considered highly polluted either except by the sampling point
416 GUA4, where the highest values for all target contaminants were observed, probably due to
417 the close location upstream of a WWTP of the town of Cordoba (328841 inhabitants). High
418 level of BPA (59.09 ± 8.12) was determined in *Luciobarbus sclater* in GUA4, which is in
419 accordance with the higher values found in river water [52] and similar to the levels found in
420 canned tuna [53] and in wild fish [15,27,45,46]. Triclosan was particularly ubiquitous in the
421 water samples of Guadalquivir, where only the fish sample GUA2 (corresponding to a rural
422 area) was free of this compound. In contrast, triclosan was only occasionally detected in
423 Llobregat and Jucar fish samples.

424 In the case of Ebro river levels found were in general higher than those found in
425 Guadalquivir and Jucar (Table 6C). Although sampling points correspond in some cases to
426 river sites located downstream urban WWTPs (Miranda de Ebro impacting EBR2, Aro
427 impacting EBR3 and EBR4 and Tudela impacting EBR5), their effect was not remarkable in
428 terms of the presence of EDCs in fish samples nor in water, probably due to the little
429 contribution of such WWTP to the overall pollution of the river in comparison to the ones in
430 Cuenca (impacting JUC2) and Córdoba (impacting GUA4). Both *Barbus graelsii* and
431 *Cyprinus carpio* were sampled at Ebro River but inter-species difference in their EDCs

432 bioaccumulation was not observed. *Silurus glanis* at EBR5 contained a great variety of
433 pollutants, which can be a consequence of the presence of the WWTP of Tudela and life
434 habits of the fish (it is a predator). However levels were not especially higher than those found
435 in the rest of fish samples at Ebro River.

436 Llobregat is the most contaminated river (higher levels of EDCs in river and fish
437 homogenates) due to the presence of important urban and industrial input in sampling point
438 LLO5 (after industrial city Martorell), LLO6 (after input of a highly polluted tributary), and
439 LLO7 (after the WWTP of Barcelona). The polluted condition of Llobregat can be highlighted
440 by the higher concentration found for TBEP comparing to the rest of fish analyzed in all
441 studied rivers. In addition, as mention above, the extremely high levels of BPA
442 (223.91 ± 11.51) in *Cyprinus carpio* in LLO5) is a matter of concern.

443

444 **4. Conclusion**

445 A simple, rapid, sensitive and efficient analytical method was developed for the
446 determination of 19 endocrine disrupting compounds from seven different chemical groups
447 (triazoles, stimulants, hormones, flame retardants, plasticizers, antibacterials, preservatives).
448 The final multi-residue procedure consisted of a QuEChERS approach (Quick, Easy, Cheap,
449 Effective, Rugged and Safe) followed by UHPLC-MS/MS analysis provided the necessary
450 sensitivity and selectivity for target analytes by monitoring two transitions per compounds. A
451 thorough evaluation of the matrix effect was performed, and thus, internal sample calibration
452 was applied to overcome such problem. The procedure was validated and is characterized by
453 good accuracy, precision and provides low quantification limits for the representative fish
454 species (*Cyprinus carpio*, *Barbus graellsii* and *Silurus glanis*); thereby, it provides a sensitive
455 and robust tool for routine analysis of EDCs in biota matrices. The developed method was
456 applied for the determination of the target EDCs in 50 samples corresponding to 12 different
457 fish species from four Mediterranean rivers (Ebro, Llobregat, Júcar and Guadalquivir). Eleven
458 out of the nineteen target EDCs were found at least once in fish homogenates. Overall
459 frequency of compounds detected varied from 2% for estrone and TCEP to 71% for TBEP
460 considering the four river basins. BPA was detected at high concentration in wild fish (at a
461 maximum of 223.91 ng/g in Llobregat River) whereas TBEP, caffeine and methyl and
462 benzylparaben were the compounds found in fish from the all four river basins.

463

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621 **Figure captions:**

622 Fig. 1. Sampling points in four Mediterranean river basins in Spain: Ebro, Llobregat, Júcar
623 and Guadalquivir

624 Fig. 2. Comparison of extraction efficiencies (%) between three extraction methods tested for
625 the target compounds (n=3)

626 Fig. 3. Comparison of extraction efficiencies (%) obtained for extraction salt of 1.5 g sodium
627 acetate, 6 g MgSO₄ combined with four different dispersive sorbents: dSPE1) 900 mg MgSO₄,
628 150 mg PSA; dSPE2) 900 mg MgSO₄, 150 mg PSA, 150 mg C18; dSPE3) 400 mg PSA, 400
629 mg 18, 400 mg GCB, 1200 mg MgSO₄; dSPE4) 150 mg PSA, 15 mg GCB, 900 mg MgSO₄

630 Fig. 4. Comparison of extraction efficiencies (%) for three different ACN/water ratios of 4:1,
631 2:1 and 4:3

632 Fig. 5. Evaluation of matrix effects for the three fish species spiked at 10 ng/g

633

634 **Table captions:**

635 Table 1. Physical-chemical properties and chemical structures of target compounds

636 Table 2. The SRM transitions and compound dependant MS parameters for target analytes

637 Table 3. Method detection (MDL) and quantification limits (MQL) in fish samples (ng/g, dry
638 weight)

639 Table 4. Mean percent recoveries (n=3) at two spiking levels for the target EDCs in fish
640 homogenates

641 Table 5. Accuracy^a and precision^b data of EDCs in fish homogenate (n=3) for representative
642 fish species (spike level 20 ng/g)

643 Table 6A. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3)
644 collected from Llobregat river (Spain)

645 Table 6B. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3)
646 collected from Júcar river (Spain)

647 Table 6C. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3)
648 collected from Ebro river (Spain)

649 Table 6D. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3)
650 collected from Guadalquivir river (Spain)

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652 Highlights:

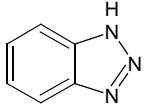
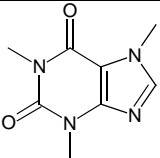
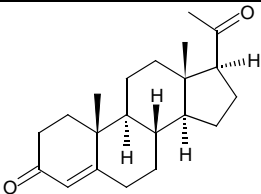
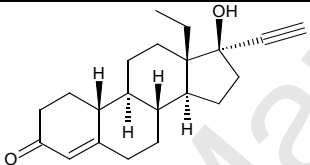
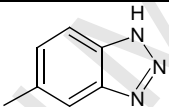
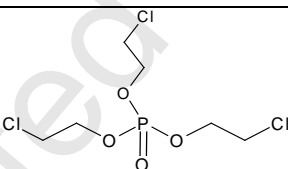
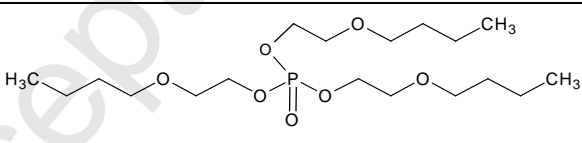
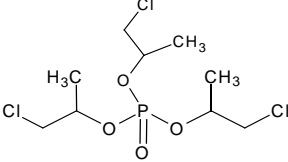
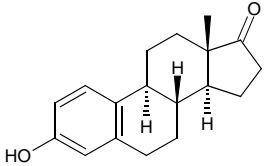
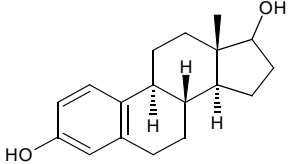
- 653 • A new, sensitive and rapid method for the determination of 19 EDCs in fish.
- 654 • The developed procedure is based on QuEChERS approach followed by UPLC-
655 MS/MS.
- 656 • Eleven EDCs were found at least once in fish homogenates from four river basins in
657 Mediterranean Area.
- 658 • TBEP, caffeine, methyl and benzyl paraben were found in fish from the four Rivers.

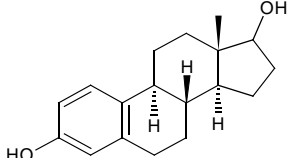
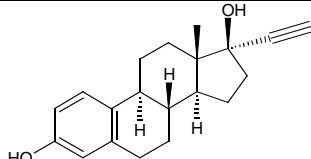
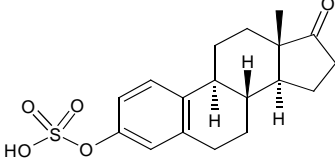
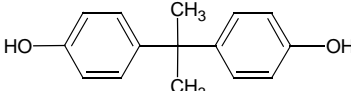
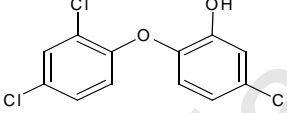
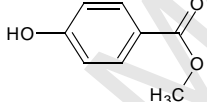
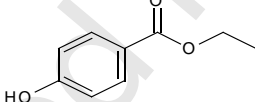
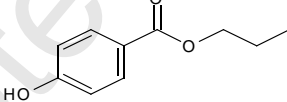
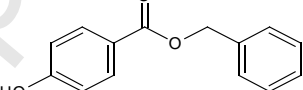
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Table 1. Physical-chemical properties and chemical structures of target compounds

| Compound | Family | Structure | pKa* | log P* | Corresponding internal standard |
|---|------------------|--|----------------------------------|--------|---------------------------------|
| 1H-benzotriazole | Triazoles |  | 0.58 8.63 | 1.30 | 1H-benzothiazole – d4 |
| Caffeine | Stimulants |  | -9.36 -0.92 | -0.55 | Caffeine –d3 |
| Progesterone | Hormones |  | -7.36 -4.82 18.92 19.56 | 4.15 | Progesterone – d8 |
| Levonorgestrel | Hormones |  | -4.73 -1.53 17.91 19.28 | 3.66 | Progesterone – d8 |
| Tolyltriazole | Triazoles |  | -2.96 -0.03 9.04 | 1.78 | 1H-benzothiazole – d4 |
| TCEP Tris(2-chloroethyl) phosphate | Flame retardants |  | -9.06 | 2.11 | Trisphenyl phosphate –d15 |
| TBEP Tris (2-butoxyethyl) phosphate | Flame retardants |  | -9.09 -4.62 -4.14 -3.66 | 3.94 | Trisphenyl phosphate –d15 |
| TCPP Tris(2-chloroisopropyl) phosphate | Flame retardants |  | -9.06 | 3.36 | Trisphenyl phosphate –d15 |
| Estrone | Hormones |  | -7.48 -5.45 10.33 19.96 | 4.31 | Estrone –d4 |
| 17β- Estradiol | Hormones |  | -5.45 -0.88 10.33 19.38 | 3.75 | 17β- Estradiol –d2 |

| | | | | | |
|-------------------------------|----------------|---|--|------|-----------------------------------|
| Estriol | Hormones |  | -5.45 -3.34 -3.16 10.33 13.62 15.16 | 2.67 | Estrone -d4 |
| 17 α -ethinylestradiol | Hormones |  | -1.66 -5.45 10.33 17.59 | 3.90 | 17 α -ethinylestradiol -d4 |
| Estrone-3-sulfate | Hormones |  | -1.75 -7.48 | 3.83 | Estrone -d4 |
| Bisphenol A | Plasticizers |  | -5.46 9.78 10.39 | 4.04 | Bisphenol A -d4 |
| Triclosan | Antibacterials |  | -9.20 -6.67 7.68 | 4.98 | Triclosan methyl-d3 ether |
| Methylparaben | Preservatives |  | -6.87 -6.06 8.50 | 1.67 | Methylparaben -d4 |
| Ethylparaben | Preservatives |  | -6.88 -6.06 8.50 | 2.03 | Methylparaben -d4 |
| Propylparaben | Preservatives |  | -6.88 -6.06 8.50 | 2.55 | Methylparaben -d4 |
| Benzylparaben | Preservatives |  | -6.89 -6.06 8.50 | 3.40 | Methylparaben -d4 |

*values given by ChemAxon; log P – partition coefficient

Table 2. The SRM transitions and compound dependant MS parameters for target analytes

| Compounds | Rt (min) | Precursor ion (m/z) | Quantification | | Confirmation | | SRM _{1/2} ratio (±SD) n=3 |
|---|----------|--------------------------|----------------|--------------|--------------|-------------|------------------------------------|
| | | | Q3 | DP/CE/CXP | Q3 | DP/CE/CXP | |
| <i>Compounds analyzed under PI mode</i> | | | | | | | |
| 1H-benzotriazole | 0.76 | 120.1 [M+H] ⁺ | 64.9 | 141/29/10 | 92.1 | 141/23/8 | 3.67 (±0.16) |
| 1H-benzotriazole -d4 | 0.75 | 124.0 [M+H] ⁺ | 69.0 | 21/29/12 | - | - | - |
| Caffeine | 0.72 | 195.0 [M+H] ⁺ | 138.0 | 86/27/20 | 42.0 | 86/63/8 | 3.16 (±0.10) |
| Caffeine -d3 | 0.72 | 198.0 [M+H] ⁺ | 138.0 | 71/27/10 | - | - | - |
| Progesterone | 2.72 | 315.0 [M+H] ⁺ | 97.1 | 86/33/8 | 109.0 | 86/39/8 | 1.09 (±0.02) |
| Progesterone -d8 | 2.70 | 323.2 [M+H] ⁺ | 100.0 | 91/29/16 | - | - | - |
| Levonorgestrel | 2.48 | 313.0 [M+H] ⁺ | 185.0 | 71/27/24 | - | - | - |
| Tolyltriazole | 1.19 | 134.1 [M+H] ⁺ | 76.9 | 41/35/10 | 78.9 | 41/27/8 | 1.66 (±0.05) |
| TCEP | 1.72 | 284.8 [M+H] ⁺ | 63.0 | 11/49/12 | 98.9 | 11/33/16 | 1.11 (±0.04) |
| TBEP | 2.79 | 399.0 [M+H] ⁺ | 299.0 | 76/19/10 | 199.0 | 76/19/10 | 1.45 (±0.04) |
| TCPP | 2.36 | 326.9 [M+H] ⁺ | 98.9 | 81/39/12 | 80.9 | 81/91/14 | 1.60 (±0.01) |
| Trisphenyl phosphate -d15 | 2.69 | 342.0 [M+H] ⁺ | 54.0 | | - | | - |
| <i>Compounds analyzed under NI mode</i> | | | | | | | |
| Estrone | 2.60 | 269.1 [M-H] ⁻ | 145.0 | -70/-48/-9 | 143.0 | -70/-76/-9 | 1.77 (±0.13) |
| Estrone -d4 | 2.60 | 273.0 [M-H] ⁻ | 145.0 | -65/-74/-7 | - | - | - |
| 17β- Estradiol | 2.59 | 271.0 [M-H] ⁻ | 145.0 | -35/-52/-9 | 183.0 | -35/-54/-9 | 1.20 (±0.05) |
| 17β- Estradiol -d2 | 2.61 | 273.0 [M-H] ⁻ | 147.0 | -35/-78/-13 | - | - | - |
| Estriol | 1.65 | 287.0 [M-H] ⁻ | 171.1 | -120/-50/-11 | 144.9 | -120/-56/-9 | 1.11 (±0.06) |
| 17α-ethinylestradiol | 2.62 | 295.1 [M-H] ⁻ | 145.0 | -50/-56/-7 | 143.0 | -50/-76/-9 | 1.02 (±0.04) |
| 17α-ethinylestradiol -d4 | 2.62 | 299.1 [M-H] ⁻ | 145.0 | -60/-76/-9 | - | - | - |
| Estrone-3-sulfate | 1.28 | 349.0 [M-H] ⁻ | 269.0 | -10/-36/-13 | 145.0 | -15/-60/-13 | 2.41 (±0.20) |
| Bisphenol A | 2.34 | 227.0 [M-H] ⁻ | 212.0 | -60/-26/-11 | 133.2 | -60/-34/-7 | 2.41 (±0.11) |
| Bisphenol A -d4 | 2.33 | 231.0 [M-H] ⁻ | 216.0 | -85/-26/-7 | - | - | - |
| Triclosan | 3.54 | 286.8 [M-H] ⁻ | 34.9 | -60/-44/-5 | - | - | - |
| Triclosan methyl-d3 ether | 3.26 | 303.0 [M-H] ⁻ | 79.9 | -55/-56/-13 | - | - | - |
| Methylparaben | 1.34 | 151.0 [M-H] ⁻ | 92.0 | -30/-20/-7 | 136.0 | -55/-20/-7 | 1.44 (±0.04) |
| Methylparaben -d4 | 1.33 | 155.0 [M-H] ⁻ | 96.1 | -65/-28/-1 | - | - | - |
| Ethylparaben | 1.79 | 165.0 [M-H] ⁻ | 92.0 | -29/-25/-7 | 136.0 | -50/-22/-7 | 1.63 (±0.03) |
| Propylparaben | 2.25 | 179.0 [M-H] ⁻ | 92.0 | -70/-32/-11 | 136.0 | -70/-22/-9 | 1.99 (± 0.10) |
| Benzylparaben | 2.65 | 227.0 [M-H] ⁻ | 92.1 | -50/-36/-7 | 135.9 | -50/-20/-9 | 1.56 (±0.06) |

Table 3. Method detection (MDL) and quantification limits (MQL) in fish samples (ng/g, dry weight)

| | MDL, ng/g d.w. | | | MQL, ng/g d.w. | | |
|-------------------------------|-------------------------|------------------------|-----------------------|-------------------------|------------------------|-----------------------|
| | <i>Barbus graellsii</i> | <i>Cyprinus carpio</i> | <i>Silurus glanis</i> | <i>Barbus graellsii</i> | <i>Cyprinus carpio</i> | <i>Silurus glanis</i> |
| Estrone | 0.35 | 0.34 | 0.06 | 1.04 | 1.02 | 0.18 |
| 17 β - Estradiol | 3.09 | 2.77 | 0.34 | 9.26 | 8.31 | 1.03 |
| Estriol | 3.00 | 2.88 | 2.00 | 9.00 | 8.64 | 6.00 |
| 17 α -ethinylestradiol | 0.62 | 0.81 | 0.60 | 1.86 | 2.44 | 1.80 |
| Estrone-3-sulfate | 0.01 | 0.02 | 0.03 | 0.02 | 0.05 | 0.09 |
| Bisphenol A | 0.01 | 0.01 | 0.003 | 0.04 | 0.03 | 0.008 |
| Triclosan | 0.27 | 0.3 | 0.25 | 0.82 | 0.9 | 0.75 |
| Methylparaben | 0.04 | 0.04 | 0.005 | 0.12 | 0.11 | 0.01 |
| Ethylparaben | 0.04 | 0.05 | 0.004 | 0.12 | 0.14 | 0.01 |
| Propylparaben | 0.004 | 0.01 | 0.002 | 0.01 | 0.02 | 0.005 |
| Benzylparaben | 0.01 | 0.02 | 0.003 | 0.04 | 0.06 | 0.01 |
| 1H-benzotriazole | 0.10 | 0.06 | 0.04 | 0.30 | 0.19 | 0.11 |
| Caffeine | 0.17 | 0.14 | 0.03 | 0.51 | 0.41 | 0.08 |
| Progesterone | 0.41 | 0.50 | 0.35 | 1.23 | 1.50 | 1.06 |
| Levonorgestrel | 0.33 | 0.35 | 0.64 | 0.99 | 1.04 | 1.92 |
| Tolyltriazole | 0.12 | 0.15 | 0.09 | 0.37 | 0.45 | 0.28 |
| TCEP | 0.10 | 0.25 | 0.13 | 0.30 | 0.75 | 0.40 |
| TBEP | 0.06 | 0.45 | 0.02 | 0.18 | 1.35 | 0.05 |
| TCPP | 0.50 | 0.09 | 0.20 | 1.50 | 0.28 | 0.60 |

Table 4. Mean percent recoveries (n=3) at two spiking levels for the target EDCs in fish homogenates

| | % Recovery \pm RSD | | | | | |
|-------------------------------|-------------------------|------------------------|-----------------------|-------------------------|------------------------|-----------------------|
| | Spiking level: 10 ng/g | | | Spiking level: 100 ng/g | | |
| | <i>Barbus graellsii</i> | <i>Cyprinus carpio</i> | <i>Silurus glanis</i> | <i>Barbus graellsii</i> | <i>Cyprinus carpio</i> | <i>Silurus glanis</i> |
| Estrone | 56.9 \pm 1.7 | 75.3 \pm 11.2 | 44.9 \pm 8.9 | 48.0 \pm 3.6 | 69.4 \pm 8.3 | 56.5 \pm 4.1 |
| 17 β - Estradiol | 47.5 \pm 12.0 | 66.4 \pm 9.5 | 68.6 \pm 3.7 | 48.7 \pm 6.8 | 54.0 \pm 7.8 | 61.1 \pm 5.9 |
| Estriol | 74.2 \pm 7.9 | 102.2 \pm 3.4 | 53.9 \pm 5.0 | 66.2 \pm 14.2 | 90.9 \pm 6.6 | 59.5 \pm 17.4 |
| 17 α -ethinylestradiol | 68.8 \pm 6.0 | 48.9 \pm 9.1 | 46.3 \pm 1.4 | 56.9 \pm 14.8 | 29.1 \pm 9.6 | 58.4 \pm 3.8 |
| Estrone-3-sulfate | 103.1 \pm 3.7 | 72.9 \pm 7.9 | 13.4 \pm 18.1 | 120.5 \pm 1.9 | 49.5 \pm 19.7 | 20.7 \pm 15.2 |
| Bisphenol A | 71.0 \pm 6.8 | 102.8 \pm 10.7 | 55.6 \pm 2.5 | 62.6 \pm 6.5 | 109.9 \pm 7.3 | 57.7 \pm 5.3 |
| Triclosan | 84.5 \pm 3.8 | 81.0 \pm 0.9 | 90.5 \pm 0.5 | 44.0 \pm 5.0 | 57.6 \pm 8.5 | 47.8 \pm 15.9 |
| Methylparaben | 73.4 \pm 11.4 | 71.4 \pm 5.9 | 38.8 \pm 3.7 | 94.5 \pm 2.0 | 89.0 \pm 7.3 | 69.2 \pm 6.7 |
| Ethylparaben | 60.3 \pm 6.3 | 77.9 \pm 6.8 | 67.0 \pm 3.6 | 108.8 \pm 16.2 | 97.6 \pm 18.8 | 71.0 \pm 4.6 |
| Propylparaben | 68.9 \pm 19.9 | 113.2 \pm 8.8 | 73.3 \pm 4.0 | 60.0 \pm 5.8 | 91.0 \pm 17.1 | 60.0 \pm 4.0 |
| Benzylparaben | 40.1 \pm 19.8 | 72.7 \pm 5.2 | 66.8 \pm 14.2 | 31.8 \pm 5.9 | 46.3 \pm 16.5 | 61.6 \pm 9.1 |
| 1H-benzotriazole | 75.0 \pm 9.6 | 69.4 \pm 2.7 | 34.2 \pm 9.1 | 65.9 \pm 8.6 | 79.8 \pm 10.6 | 62.5 \pm 11.3 |
| Caffeine | 96.8 \pm 5.8 | 72.9 \pm 6.5 | 65.9 \pm 10.5 | 95.6 \pm 5.1 | 73.4 \pm 6.7 | 82.0 \pm 6.8 |
| Progesterone | 71.8 \pm 11.1 | 60.7 \pm 7.1 | 59.2 \pm 4.9 | 55.1 \pm 5.9 | 75.0 \pm 8.9 | 58.9 \pm 1.8 |
| Levonorgestrel | 81.7 \pm 14.5 | 89.7 \pm 8.2 | 75.5 \pm 13.6 | 77.5 \pm 4.9 | 100.6 \pm 8.4 | 63.2 \pm 3.5 |
| Tolyltriazole | 89.0 \pm 9.3 | 63.2 \pm 4.9 | 78.1 \pm 8.6 | 58.7 \pm 7.0 | 67.4 \pm 9.3 | 79.6 \pm 10.8 |
| TCEP | 95.5 \pm 4.1 | 69.6 \pm 10.2 | 68.5 \pm 4.1 | 116.3 \pm 6.2 | 125.1 \pm 4.1 | 109.2 \pm 7.7 |
| TBEP | 51.7 \pm 4.7 | 85.4 \pm 8.8 | 40.3 \pm 14.5 | 76.6 \pm 9.0 | 102.6 \pm 0.6 | 65.5 \pm 11.5 |
| TCPP | 74.3 \pm 8.9 | 100.5 \pm 29.2 | 64.0 \pm 10.3 | 103.8 \pm 3.4 | 104.4 \pm 0.2 | 83.7 \pm 10.1 |

Table 5. Accuracy^a and precision^b data of EDCs in fish homogenate (n=3) for representative fish species (spike level 20 ng/g)

| Compound | <i>Barbus graellsii</i> | | <i>Cyprinus carpio</i> | | <i>Silurus glanis</i> | |
|-------------------------------|-------------------------|------|------------------------|------|-----------------------|------|
| | Bias (%) | RSD% | Bias (%) | RSD% | Bias (%) | RSD% |
| Estrone | 0.72 | 3.65 | 2.90 | 5.90 | 10.37 | 1.62 |
| 17 β - Estradiol | -5.65 | 0.46 | -8.78 | 1.22 | 9.90 | 4.41 |
| Estriol | 6.82 | 0.85 | 12.40 | 2.92 | 6.60 | 1.44 |
| 17 α -ethinylestradiol | 4.92 | 8.12 | 8.37 | 4.63 | 12.70 | 4.14 |
| Estrone-3-sulfate | 15.39 | 2.65 | 15.64 | 1.27 | 13.34 | 1.17 |
| Bisphenol A | 1.76 | 2.33 | -5.51 | 12.2 | 11.76 | 3.79 |
| Triclosan | 4.93 | 9.88 | 15.15 | 4.23 | 10.37 | 0.97 |
| Methylparaben | -5.90 | 1.00 | 7.04 | 0.65 | 5.31 | 0.79 |
| Ethylparaben | -1.67 | 1.50 | 3.33 | 1.75 | 11.99 | 0.57 |
| Propylparaben | -4.88 | 0.18 | 18.49 | 2.41 | 15.35 | 1.06 |
| Benzylparaben | -2.96 | 0.78 | 8.42 | 1.99 | 6.07 | 0.32 |
| 1H-benzotriazole | -1.79 | 9.65 | -12.38 | 3.67 | -1.96 | 2.20 |
| Caffeine | -7.35 | 2.58 | -7.16 | 1.08 | 6.65 | 0.98 |
| Progesterone | -3.07 | 2.09 | 5.99 | 1.69 | 15.26 | 0.56 |
| Levonorgestrel | -17.01 | 1.85 | 13.56 | 2.10 | -3.83 | 0.46 |
| Tolyltriazole | -2.91 | 0.82 | -1.23 | 2.79 | 7.21 | 1.64 |
| TCEP | 11.89 | 1.25 | 15.67 | 2.38 | 17.05 | 0.18 |
| TBEP | 9.27 | 1.31 | 17.64 | 1.79 | 15.94 | 1.60 |
| TCPP | 2.04 | 0.28 | 20.24 | 1.42 | 16.45 | 3.88 |

^a Accuracy expressed as the percentage value of the bias between the theoretical and calculated concentrations

^b Precision expressed as relative standard deviation (RSD (%))

Table 6A. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3) collected from Llobregat river (Spain)

| Compound | Concentration range \pm SD (ng/g, d.w.) | | | | | | | | |
|---------------|--|----------------------|--|------------------------|-------------------|--------------------|------------------|-------------------------|------------------|
| | <i>Barbus graellsii</i> | | | <i>Cyprinus carpio</i> | | | | <i>Lepomis gibbosus</i> | |
| | Llobregat River – Sampling point | | | | | | | | |
| | LLO3 | LLO4 | LLO6 | LLO3 | LLO4 | LLO5 | LLO6 | LLO7 | LLO3 |
| Bisphenol A | <MDL | <MDL | <MDL | <MDL | <MDL | 223.91 \pm 11.51 | <MDL | <MDL | <MDL |
| Triclosan | <MDL | <MDL | <MDL | <MDL | <MDL | 1.25 \pm 0.09 | <MDL | <MDL | <MDL |
| Methylparaben | 2.56 \pm 0.21 | <MDL | 62.85 \pm 6.52 (A); 33.65 \pm 3.70 (J) | 0.80 \pm 0.05 | 0.66 \pm 0.04 | 1.68 \pm 0.24 | 0.63 \pm 0.10 | 2.53 \pm 0.38 | 9.08 \pm 1.06 |
| Propylparaben | <MDL | <MDL | 3.48 \pm 0.58 (A); 0.19 \pm 0.04 (J) | <MDL | <MDL | <MDL | <MDL | <MDL | 0.64 \pm 0.13 |
| Benzylparaben | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 0.35 \pm 0.02 |
| Caffeine | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 1.27 \pm 0.05 |
| Tolytriazole | <MDL | <MDL | <MDL | <MDL | <MDL | 10.18 \pm 3.94 | <MDL | <MDL | <MDL |
| TBEP | 52.96 \pm 19.13 (A); 31.10 \pm 4.33 (J) | 34.96 \pm 5.47 (A) | 28.13 \pm 6.16 (A); 47.18 \pm 22.65 (J) | 24.47 \pm 10.94 | 30.70 \pm 17.80 | 38.13 \pm 4.89 | 40.39 \pm 3.69 | 34.85 \pm 19.06 | 27.27 \pm 9.12 |

Table 6B. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3) collected from Júcar river (Spain)

| Compound | Concentration range \pm SD (ng/g, d.w.) | | | | | | | | | | | | | | | | |
|---------------|---|------------------|--------------------|------------------------|------|--------------------------------------|------------------------------|-------|-----------------|-------------------------|------|-------------------------|------|--------------------------|------------------|-------------------------|-----------------------------|
| | <i>Salmo trutta</i> | | <i>Gobio gobio</i> | | | <i>Pseudochondrostoma willkommii</i> | <i>Micropterus salmoides</i> | | | <i>Lepomis gibbosus</i> | | <i>Aburnus alburnus</i> | | <i>Anguilla anguilla</i> | | <i>Barbus graellsii</i> | <i>Luciobarbus sclateri</i> |
| | Júcar River – Sampling point | | | | | | | | | | | | | | | | |
| | JUC1 | JUC2 | JUC2 | JUC4 | JUC6 | JUC2 | JUC4 | JUC5 | JUC6 | JUC5 | JUC6 | JUC5 | JUC6 | JUC5 | JUC6 | JUC6 | JUC6 |
| Triclosan | <MDL | <MDL | <MDL | <MDL | 0.62 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| Methylparaben | <MDL | 84.69 \pm 6.58 | <MDL | <MDL | <MDL | <MDL | 4.45 \pm 0.44 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 2.97 \pm 0.13 | <MDL | <MDL | <MDL |
| ylparaben | 0.82 (A); 0.78 (J) | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| ylparaben | <MDL | 7.43 \pm 0.69 | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL |
| ylparaben | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 0.54 | 0.38 | <MDL | <MDL | 0.33 \pm 0.01 | 0.50 \pm 0.04 | <MDL | <MDL |
| eine | 20.49 (A); 11.71 (J) | 21.40 \pm 0.95 | 2.75 | 9.95 (A); 16.20 (J) | 1.91 | 12.91 | 9.27 \pm 0.54 | 10.55 | <MDL | 5.83 | 1.15 | <MDL | 1.93 | 9.86 \pm 1.19 | 3.54 \pm 0.45 | 4.35 \pm 0.78 | 2.36 \pm 0.36 |
| IP | <MDL | 10.60 \pm 5.69 | <MDL | <MDL | <MDL | <MDL | 7.79 \pm 5.97 | <MDL | 8.01 \pm 0.24 | <MDL | <MDL | <MDL | <MDL | 9.16 \pm 4.10 | 11.07 \pm 2.28 | 3.72 \pm 1.08 | 9.88 \pm 3.21 |

Table 6C. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3) collected from Ebro river (Spain)

| Compound | Concentration range \pm SD (ng/g, d.w.) | | | | | | | | | | |
|----------------------|---|--|---|----------------------|---|------------------------|------------------|------------------|------------------|-----------------------|------------------|
| | <i>Barbus graellsii</i> | | | | | <i>Cyprinus carpio</i> | | | | <i>Silurus glanis</i> | |
| | Ebro River – Sampling point | | | | | | | | | | |
| | OCA | EBR2 | EBR3 | EBR4 | EBR5 | EBR2 | EBR3 | EBR4 | EBR5 | EBR4 | EBR5 |
| Estrone | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 1.99 \pm 0.19 | <MDL | <MDL | <MDL | <MDL |
| Methylparaben | <MDL | <MDL | <MDL | 1.16 \pm 0.09 | 1.71 \pm 0.13 (J); 1.58 \pm 0.28 (A) | 3.41 \pm 0.59 | 2.98 \pm 0.51 | 2.56 \pm 0.44 | <MDL | 3.23 \pm 0.28 | <MDL |
| Benzylparaben | <MDL | <MDL | 0.37 \pm 0.03 (J); 0.35 \pm 0.02 (A) | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 0.40 \pm 0.03 |
| Caffeine | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 4.14 \pm 0.40 |
| Tolyltriazole | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 1.25 \pm 0.29 |
| TCEP | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | <MDL | 5.11 \pm 0.69 |
| TBEP | 29.18 \pm 11.21 (J); 9.22 \pm 5.86 (A) | 30.99 \pm 7.76 (J); 6.93 \pm 4.06 (A) | 17.48 \pm 10.77 (J); 5.61 \pm 2.02 (A) | 24.25 \pm 7.29 (A) | 17.06 \pm 4.76 (J); 20.62 \pm 5.75 (A) | 23.32 \pm 6.11 | 20.59 \pm 0.93 | 29.12 \pm 5.90 | 23.14 \pm 6.78 | 30.29 \pm 4.35 | 18.98 \pm 8.14 |

Table 6D. Mean concentration (\pm SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3) collected from Guadalquivir river (Spain)

| Compound | Concentration range \pm SD (ng/g, d.w.) | | | | |
|----------------------|---|------|------------------|------------------|------------------|
| | <i>Luciobarbus sclateri</i> | | | | |
| | Guadalquivir River – Sampling point | | | | |
| | GUA1 | GUA2 | GUA3 | GUA4 | GUA5 |
| Bisphenol A | <MDL | <MDL | <MQL | 59.09 \pm 8.12 | <MQL |
| Triclosan | 1.98 \pm 0.29 | <MDL | 17.41 \pm 1.81 | 16.77 \pm 1.43 | 13.85 \pm 1.90 |
| Methylparaben | 2.81 \pm 0.07 | <MDL | 0.97 \pm 0.12 | 24.45 \pm 1.38 | <MDL |
| Propylparaben | <MDL | <MDL | <MDL | 0.63 \pm 0.06 | <MDL |
| Benzylparaben | <MDL | <MDL | 0.42 \pm 0.06 | 0.33 \pm 0.01 | 0.37 \pm 0.01 |
| Caffeine | 1.68 \pm 0.08 | 0.56 | 1.34 \pm 0.14 | 15.22 \pm 1.72 | <MQL |
| TBEP | 13.49 \pm 6.06 | <MDL | 15.45 \pm 7.01 | 12.83 \pm 4.41 | 20.09 \pm 6.99 |

(A) – adult; (J) – juvenile

Figure 1

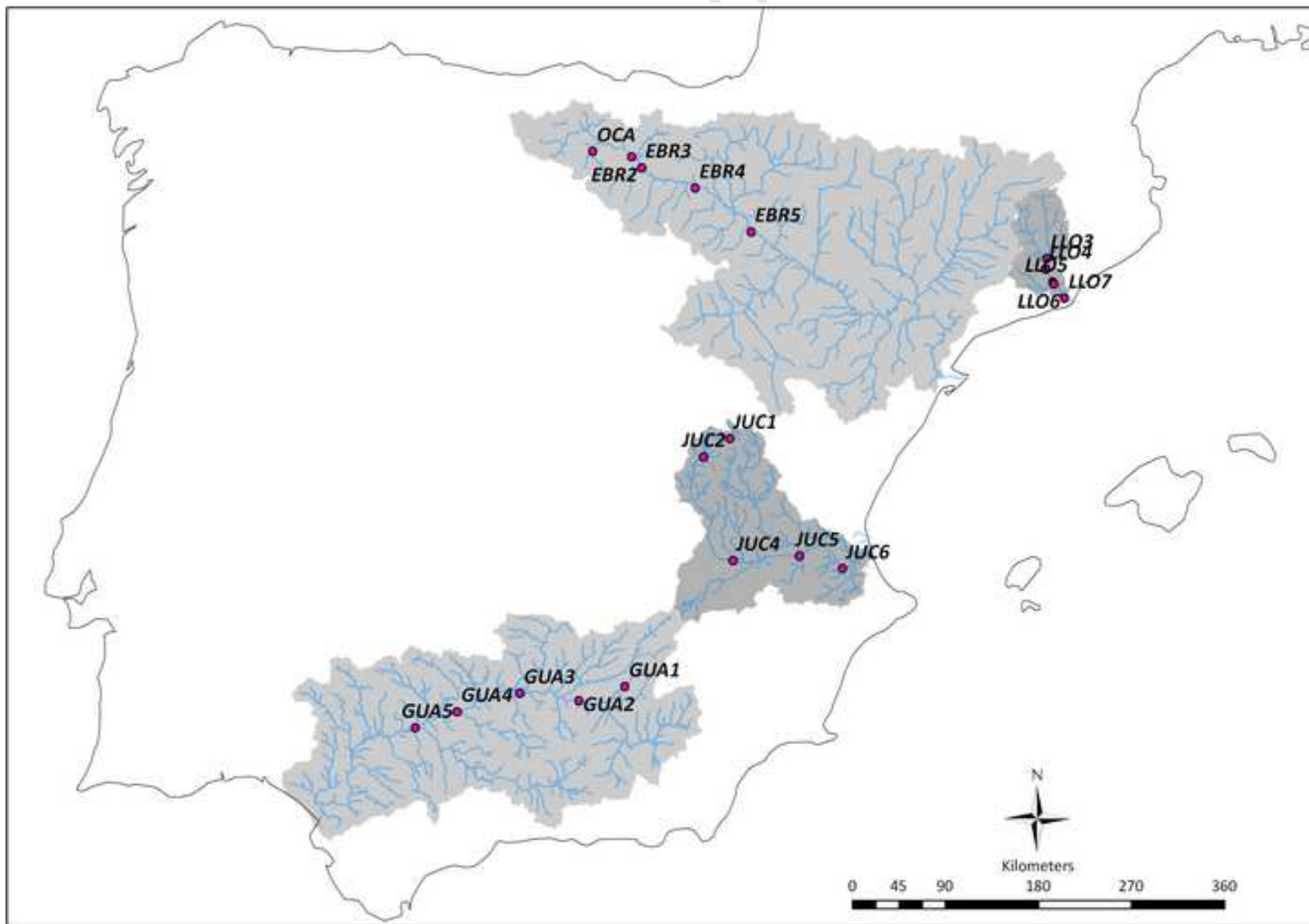


Figure 2

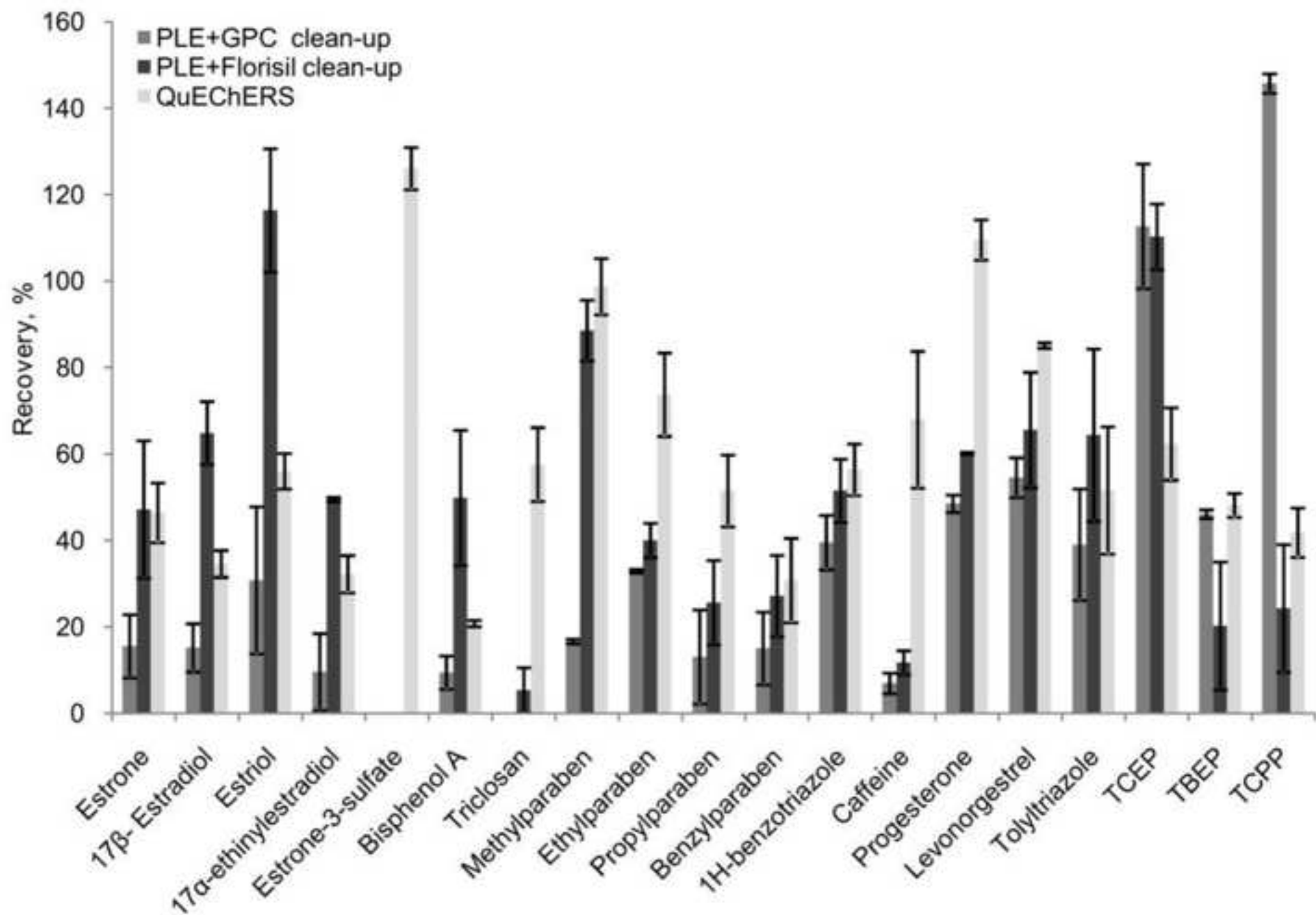


Figure 3

Script

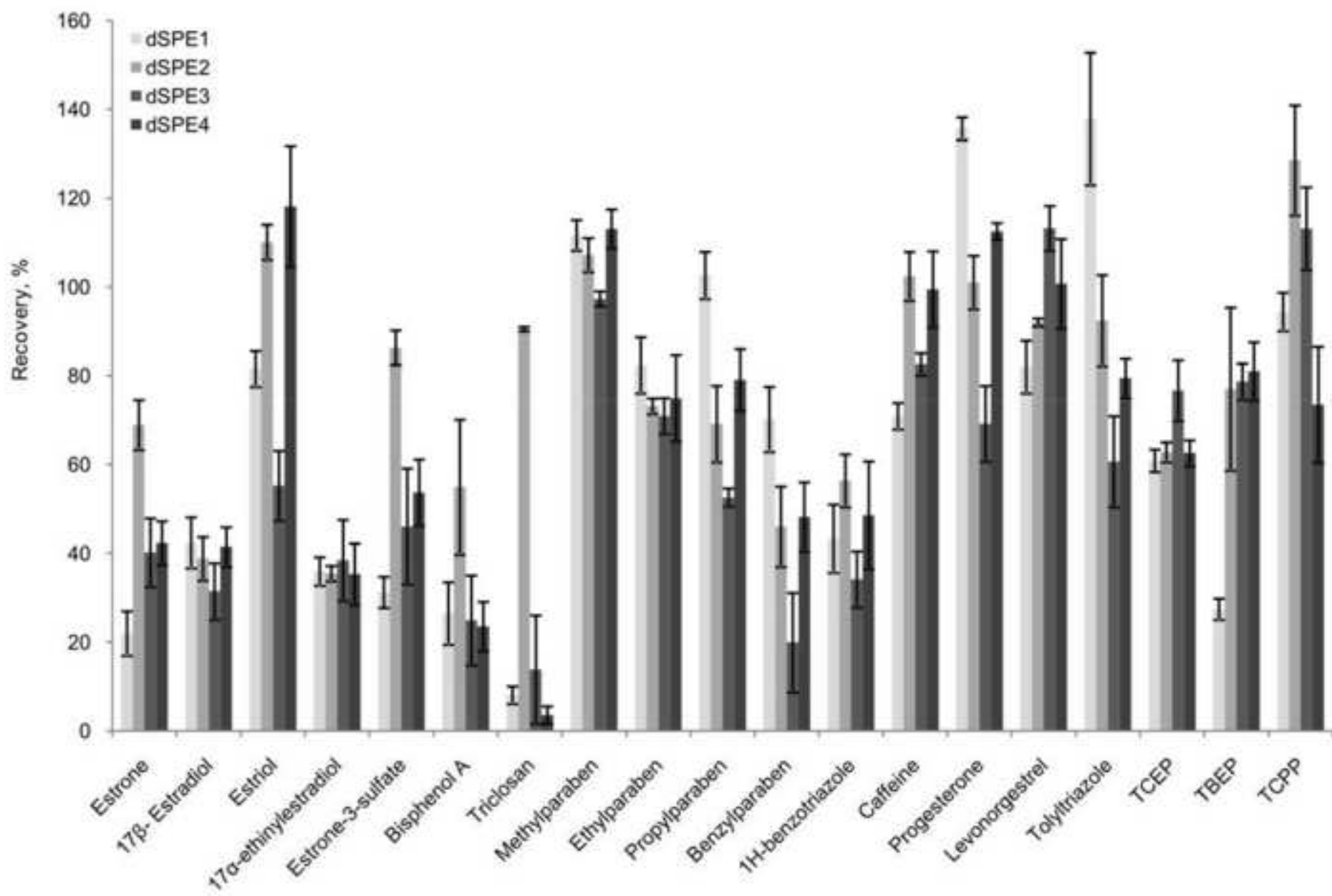


Figure 4

scrip

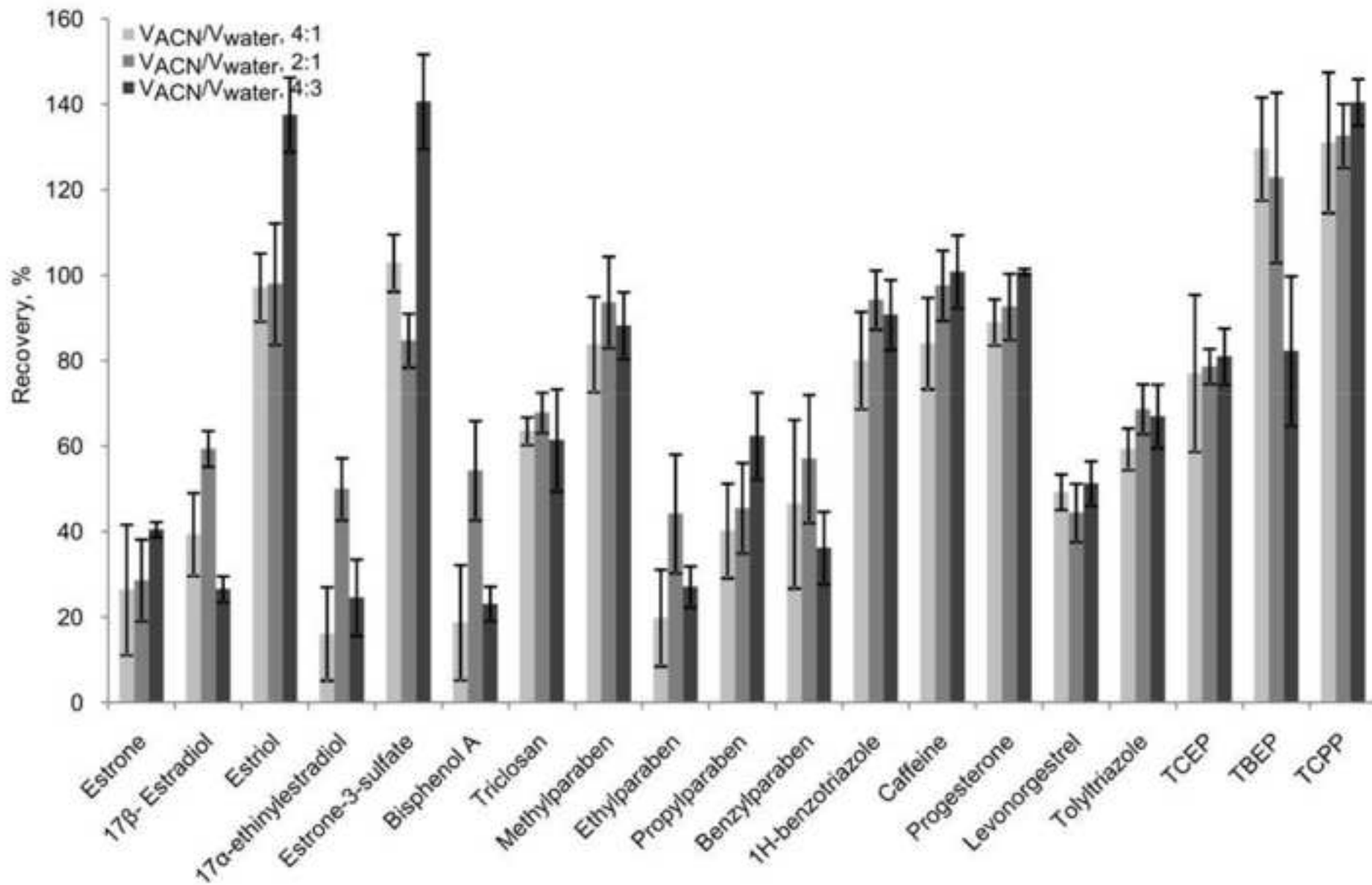


Figure 5

