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

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

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

1. Introduction

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

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

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

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

In light of the lack of multi-residue analytical methods for the determination of several EDCs in very complex samples and the interest of analyzing them in aquatic biota, the aim of the present work was to develop a multi-residue procedure for the simultaneous determination of 19 main concerned EDCs present at ultra-trace levels in fish homogenates. Different sample preparation procedures were tested; and the most appropriate methodology was based on QuEChERS extraction followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. This paper describes the comparison of different sample preparation techniques and also the optimization and validation of the analytical method of choice. According to our knowledge, this is the simplest and most rapid procedure that has been successfully applied to fish samples allowing the simultaneous determination of 19 multi-class EDCs (19 compounds from different classes: triazoles, stimulants, hormones, flame retardants, plasticizers, antibacterials, preservatives). This methodology is competitive in terms of number of EDCs determined in one analytical cycle, sensitivity, rapidity (is faster than the other methods published) and efficiency. The QuEChERS-LC-MS/MS methodology is applied for the first time for the simultaneous determination of trace levels of 19 EDCs in fish and provides good recoveries and low limits of quantification and was further applied to determine the presence of the target compounds in wild fish collected during a monitoring campaign in four different river basins in Mediterranean area.

2. Materials and methods

2.1. Standards and reagents

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

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

2.2. Sample collection and preservation

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

2.3. *Optimization of sample preparation step*

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

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

The second extraction method was PLE followed by Florisil clean-up similarly to Gorga *et al.* [41] method. Analytes were extracted with a mixture of acetone/MeOH/H_2O (1:2:1 v/v/v) in 4 cycles of 5 min each at 50^{o}C using 1 g of a fish homogenate. Final extracts were evaporated to dryness under a stream of nitrogen, reconstituted in 8 mL of ACN and subjected to clean-up procedure. Purification was carried out with Florisil cartridges (Agilent Technologies) which were conditioned with 5 mL portion of hexane and 5 mL of ACN. Later acetonitrile extracts were passed through a sorbent, followed by 2 mL of ACN. All was collected and evaporated to dryness.

The third approach based on QuEChERS (QuEChERS Kits, Agilent Technologies) involved two steps, extraction with acetonitrile in aqueous conditions followed by the application of specific salt (4g $MgSO_4$, 1 g NaCl) used for salting out of water from the sample and to induce liquid-liquid partitioning; and purification with dispersive solid phase extraction (dSPE) using sorbent mixture (900 mg MgSO4, 150 mg PSA (primary and secondary amine exchange material), 150 mg C18). Once QuEChERS was chosen as the procedure which provided the best results for target compounds, the best conditions were further optimized for fish homogenate. Three extraction salts: (I) 4g $MgSO_4$, 1 g NaCl; (II) 1.5 g sodium acetate, 6 g $MgSO_4$; (III) 4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate dehydrate, 0.5 g disodium hydrogencitrate sesquihydrate, and four dSPE sorbents: (I) 900 mg $MgSO_4$, 150 mg PSA; (II) 900 mg $MgSO_4$, 150 mg PSA, 150 mg C18; (III) 400 mg PSA, 400 mg C18, 400 mg GCB (graphitized carbon black), 1200 mg $MgSO_4$; (IV) 150 mg PSA, 15 mg GCB, 900 mg $MgSO_4$, were tested in different combinations. After choosing the best extraction salt – dSPE sorbent pair other parameters such as sample weight (0.5g, 1g, 1.5g) and a volume of ACN added to reach different V_{ACN}/V_{water} ratios of 4:1, 2:1 and 4:3, were optimized. Also a different approach, which includes application of hexane as a purification solvent instead of typical dSPE sorbent, similar to the one proposed by Pouech *et al.* [1] was tested. For hexane purification, a specific volume of hexane was added right after ACN portion leading to a V_{ACN}/V_{hexane} ratio of 2:1, vortexed for 30 s and as follows in the overall procedure excluding the dSPE step.

All extracts after drying were reconstituted in 0.5 mL of MeOH/H_2O (1:1, v/v) and finally, 10 µL of IS mixture was added and vortexed with the sample thoroughly before LC-MS/MS analysis.

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

2.4. UHPLC-MS/MS analysis

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

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

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

3. Results and discussion

3.1. Optimization of sample preparation step for fish homogenates

Three preliminary sample pretreatment protocols based on PLE extraction with GPC clean-up, PLE extraction with Florisil clean-up and QuEChERS were compared in term of extraction efficiencies for the 19 endocrine disrupting compounds. Fish samples are characterized by high lipid content, from 10-15 % (*Cyprinus carpio* and *Barbus graellsii) to*, 25 % (*Silurus glanis* - up to) [40], and therefore, in addition to the corresponding extraction step, it was necessary to include a purification step through either GPC analysis or Florisil sorbents. The extraction efficiencies for PLE with GPC clean-up was considered as an inefficient method as only five out of the nineteen compounds had recoveries higher than 40% (Fig. 2). In addition, the compounds, estrone-3-sulfate and triclosan were not recovered at all. Results obtained for PLE with Florisil clean-up, allowed the extraction of most of the compounds (Fig. 2); however, estrone metabolite was still not extracted from the matrix. Although the extraction of compounds from solid matrices should be better in PLE, as it is assisted by high temperature and pressure, it also co-extracts other matrix components, which may not be sufficiently removed during the purification step leading to high matrix effects. QuEChERS method, which includes micro-scale extraction and purification with dispersive solid phase extraction (dSPE), was finally chosen as the most efficient method: QuEChERS approach allowed the simultaneous extraction of all target compounds and provided satisfactory recoveries (Fig. 2) surpassing 40% for the most relevant analytes and low values of relative standard deviation (RSD%). Application of QuEChERS may cause co-extraction of non-target compounds as well and therefore, it was crucial to perform further optimization tests with different extraction salts and sorbents used for dSPE. Each extraction salt (3 different in total) was tested with the 4 purification sorbents selected, giving in overall twelve pairs. The best results were obtained for the combination of the extraction salt composed with 1.5 g sodium acetate, 6 g $MgSO_4$, thus only combinations based on this salt with different sorbents are presented in the Fig. 3. As it can be seen, the second dSPE sorbent (900 mg $MgSO_4$, 150 mg PSA, 150 mg C18) gave the most satisfactory results since this sorbents mixture is dedicated to samples with high lipid content [Technologies, 2011 #64]. The recoveries exceed 50% for most of the target compounds and low RSD% values <18% were achieved. Furthermore, only this particular dSPE sorbent provided much higher extraction efficiency for such relevant compounds as BPA, triclosan, estrone and its metabolite estrone-3-sulfate. The purification with hexane instead of dSPE as suggested by Pouech et al. [1] was tested as well; however, high matrix effects were observed leading to the final optimal recovery for only five out of nineteen EDCs.

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

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

3.2. Matrix effect

A significant drawback in the MS analysis performed with electrospray (ESI) as ionization technique is the appearance of matrix effect, especially when analyzing complex matrices such as fish. This occurs due to the high sensitivity of ESI source to different components present in the matrix, which can lead to signal suppression or enhancement, thereby leading to false quantitative results. A thorough evaluation of matrix effect (ME%) for fish homogenate was thus performed by comparing the peak area of the target compound in fish extract spiked at 10 ng/g (after previous subtraction of the peak area of the analyte present in the extract) with the peak area of the analyte in the solvent (MeOH/H_2O 1:1 v/v) at the same concentration level. The percentage of matrix effect was then calculated according to the equation: $ME\% = (A_{matrix}/A_{solvent} - 1) \times 100$ [28]. Calculations were performed in triplicate for three fish species (*Cyprinus carpio, Barbus graellsii* and *Silurus glanis*) and the values obtained are presented in Fig. 5. The results for the different fish species indicate that ion suppression was observed for all EDCs. The lowest ME% was observed for estrone-3-sulfate (-11.6% for *Silurus glanis*); however, for the other compounds matrix effects were high up to 98% for estrone, 17β-estradiol and triclosan for *Barbus graellsii*.

In order to overcome ion suppression different approaches that should include the variability of the matrices can be undertaken, such as selective and efficient purification of the sample prior to analysis. However, such approach is not always appropriate and may lead to analyte loss or increase of analysis time. Different, reliable and effective strategies described in the literature are based on appropriate calibration methods, such as standard addition, internal standard with isotopically labeled standards or matrix-matched calibration [42]. Although the best choice is the application of standard addition, it is a very time-consuming approach due to the high amount number of different samples to process. Internal standard calibration, on the other hand, is based on the addition to the sample extract of isotopically labeled compounds that are structurally similar to the target analytes. This allows the correction of the matrix effect since internal standard undergoes the same interferences as the analytes. However, only ten isotopic analogues out of the nineteen target compounds were available, and thus, this method did not seem the most appropriate for an accurate determination of the whole set of target compounds. A good alternative is the application of matrix-matched calibration, but it requires matrices (similar to the one analyzed) free from the target compounds, which was not possible in this case. Therefore, a different strategy previously applied by Stüber *et al.* [43], called internal sample calibration, was adopted. This approach combines advantages of both, matrix-matched and internal standard calibration, enabling the correction of the matrix effects for all the target compounds. For this purpose, calibration curves were prepared in fish extracts for each fish species, with addition of available isotopically labeled compounds and were presented as a dependence of the ratio of a peak area of an analyte and a peak area of an internal standard to an analyte concentration. The internal sample calibration prepared in the matrix which is consistent or similar to the fish species analyzed was considered as the best approach for the determination of EDCs in biota samples.

3.3. Method validation

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

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

Total recoveries were calculated for two spiking levels, 10 and 100 ng/g, which were set as the lower and higher level of expected EDCs levels in fish samples based on literature. Recoveries were determined for the final sample preparation method for fish homogenates and were calculated by internal sample calibration. Results, summarized in Table 4, were obtained for the three representative species *Cyprinus carpio, Barbus graellsii* and *Silurus glanis*. Recoveries ranged from 40.1 ± 19.8 (benzylparaben) to 103.1 ± 3.7 (estrone-3-sulfate) for *Barbus graellsii*; from 48.9 ± 9.1 (17α-ethinylestradiol) to 113.2 ± 8.8 (propylparaben) for *Cyprinus carpio*; from 34.2 ± 9.1 (1H-benzotriazole) to 90.5 ± 0.5 (triclosan) for *Silurus* *glanis* (except for estrone-3-sulfate) for lower spiking level. Recoveries higher than 40% were accepted [27,44] since the determination of EDCs in biota matrices is a challenging issue for the following reasons i) EDCs belong to different classes, thus they differ in physic-chemical properties (e.g. lipophilicity, log P, pKa); ii) biota samples contain a high amount of interferences which have a significant influence at sample preparation step as well as LC-MS/MS analysis (e.g. high lipid content and non-target compounds which are co-extracted). However, recoveries for some compounds in case of specific species (e.g. estrone recoveries for *Silurus glanis*) were lower for higher spiking level (100 ng/g). That phenomenon can be explained by the decrease of extraction and purification efficiency when working at high concentrations. This is due to the fact that the capacity of solvent during extraction (first step) and sorbents in dSPE during purification (second step) is limited.

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

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

3.4. Application to real samples

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

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

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

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

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

In the case of Ebro river levels found were in general higher than those found in Guadalquivir and Jucar (Table 6C). Although sampling points correspond in some cases to river sites located downstream urban WWTPs (Miranda de Ebro impacting EBR2, Aro impacting EBR3 and EBR4 and Tudela impacting EBR5), their effect was not remarkable in terms of the presence of EDCs in fish samples nor in water, probably due to the little contribution of such WWTP to the overall pollution of the river in comparison to the ones in Cuenca (impacting JUC2) and Córdoba (impacting GUA4). Both *Barbus graelsii* and *Cyprinus carpio* were sampled at Ebro River but inter-species difference in their EDCs bioaccumulation was not observed. *Silurus glanis* at EBR5 contained a great variety of pollutants, which can be a consequence of the presence of the WWTP of Tudela and life habits of the fish (it is a predator). However levels were not especially higher than those found in the rest of fish samples at Ebro River.

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

4. Conclusion

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

Acknowledgements

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Figure captions:

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

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

Fig. 3. Comparison of extraction efficiencies (%) obtained for extraction salt of 1.5 g sodium acetate, 6 g $MgSO_4$ combined with four different dispersive sorbents: dSPE1) 900 mg $MgSO_4$, 150 mg PSA; dSPE2) 900 mg $MgSO_4$, 150 mg PSA, 150 mg C18; dSPE3) 400 mg PSA, 400 mg 18, 400 mg GCB, 1200 mg $MgSO_4$; dSPE4) 150 mg PSA, 15 mg GCB, 900 mg $MgSO_4$

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

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

Table captions:

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

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

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

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

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

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

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

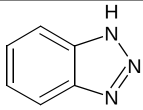
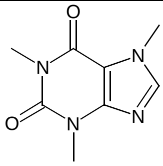
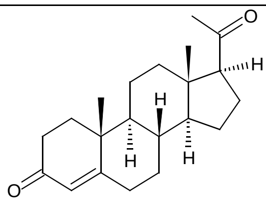
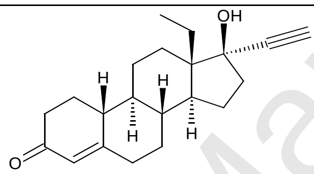
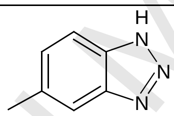
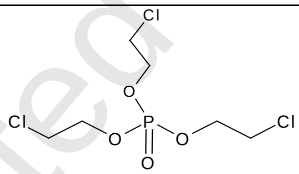
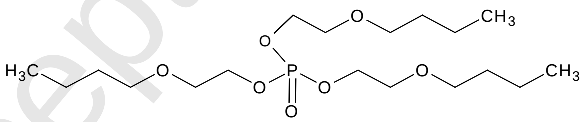
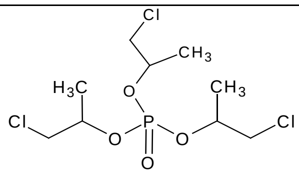
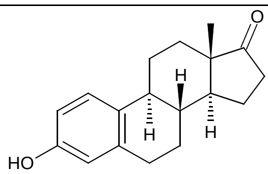
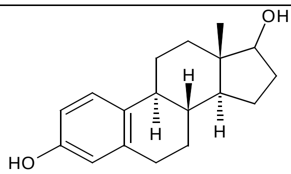
Table 6C. Mean concentration (± SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3) collected from Ebro river (Spain)

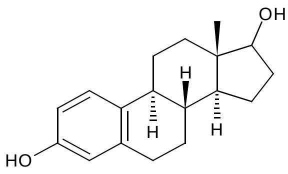
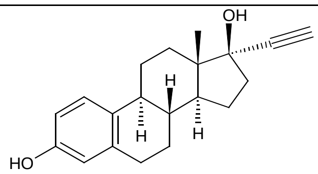
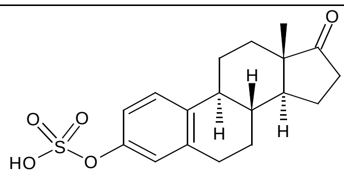
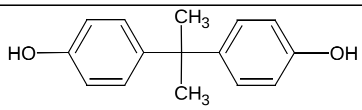
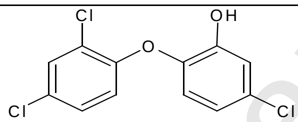
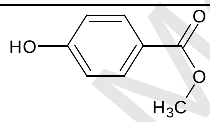
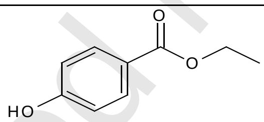
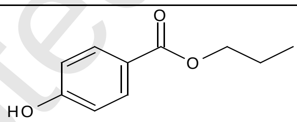
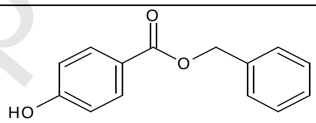
Table 6D. Mean concentration (± SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3) collected from Guadalquivir river (Spain)

Highlights:

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

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
17α-ethinylestradiol	Hormones		-1.66 -5.45 10.33 17.59	3.90	17α-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

			Quantification		Confirmation		
Compounds	**Rt (min)**	**Precursor ion (m/z)**	**Q3**	**DP/CE/CXP**	**Q3**	**DP/CE/CXP**	**$SRM_{1/2}$ ratio (±SD) n=3**
Compounds analyzed under PI mode							
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		-		-
Compounds analyzed under NI mode							
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.		
	Barbus graellsii	*Cyprinus carpio*	*Silurus glanis*	*Barbus graellsii*	*Cyprinus carpio*	*Silurus glanis*
Estrone	0.35	0.34	0.06	1.04	1.02	0.18
17β- 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
17α-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 ± RSD					
	Spiking level: 10 ng/g			Spiking level: 100 ng/g		
	Barbus graellsii	*Cyprinus carpio*	*Silurus glanis*	*Barbus graellsii*	*Cyprinus carpio*	*Silurus glanis*
Estrone	56.9 ± 1.7	75.3 ± 11.2	44.9 ± 8.9	48.0 ± 3.6	69.4 ± 8.3	56.5 ± 4.1
17β- Estradiol	47.5 ± 12.0	66.4 ± 9.5	68.6 ± 3.7	48.7 ± 6.8	54.0 ± 7.8	61.1 ± 5.9
Estriol	74.2 ± 7.9	102.2 ± 3.4	53.9 ± 5.0	66.2 ± 14.2	90.9 ± 6.6	59.5 ± 17.4
17α-ethinylestradiol	68.8 ± 6.0	48.9 ± 9.1	46.3 ± 1.4	56.9 ± 14.8	29.1 ± 9.6	58.4 ± 3.8
Estrone-3-sulfate	103.1 ± 3.7	72.9 ± 7.9	13.4 ± 18.1	120.5 ± 1.9	49.5 ± 19.7	20.7 ± 15.2
Bisphenol A	71.0 ± 6.8	102.8 ± 10.7	55.6 ±2.5	62.6 ± 6.5	109.9 ± 7.3	57.7 ± 5.3
Triclosan	84.5 ± 3.8	81.0 ± 0.9	90.5 ± 0.5	44.0 ± 5.0	57.6 ± 8.5	47.8 ± 15.9
Methylparaben	73.4 ± 11.4	71.4 ± 5.9	38.8 ± 3.7	94.5 ± 2.0	89.0 ± 7.3	69.2 ± 6.7
Ethylparaben	60.3 ± 6.3	77.9 ± 6.8	67.0 ±3 6	108.8 ± 16.2	97.6 ± 18.8	71.0 ± 4.6
Propylparaben	68.9 ± 19.9	113.2 ± 8.8	73.3 ± 4.0	60.0 ± 5.8	91.0 ± 17.1	60.0 ± 4.0
Benzylparaben	40.1 ± 19.8	72.7 ± 5.2	66.8 ± 14.2	31.8 ± 5.9	46.3 ± 16.5	61.6 ± 9.1
1H-benzotriazole	75.0 ± 9.6	69.4 ± 2.7	34.2 ± 9.1	65.9 ± 8.6	79.8 ± 10.6	62.5 ± 11.3
Caffeine	96.8 ± 5.8	72.9 ± 6.5	65.9 ± 10.5	95.6 ± 5.1	73.4 ± 6.7	82.0 ± 6.8
Progesterone	71.8 ± 11.1	60.7 ± 7.1	59.2 ± 4.9	55.1 ± 5.9	75.0 ± 8.9	58.9 ± 1.8
Levonorgestrel	81.7 ± 14.5	89.7 ± 8.2	75.5 ± 13.6	77.5 ± 4.9	100.6 ± 8.4	63.2 ± 3.5
Tolyltriazole	89.0 ± 9.3	63.2 ± 4.9	78.1 ± 8.6	58.7 ± 7.0	67.4 ± 9.3	79.6 ± 10.8
TCEP	95.5 ± 4.1	69.6 ± 10.2	68.5 ± 4.1	116.3 ± 6.2	125.1 ± 4.1	109.2 ± 7.7
TBEP	51.7 ± 4.7	85.4 ± 8.8	40.3 ± 14.5	76.6 ± 9.0	102.6 ± 0.6	65.5 ± 11.5
TCPP	74.3 ± 8.9	100.5 ± 29.2	64.0 ± 10.3	103.8 ± 3.4	104.4 ±0.2	83.7 ± 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	*Barbus graellsii*		*Cyprinus carpio*		*Silurus glanis*	
	Bias (%)	RSD%	Bias (%)	RSD%	Bias (%)	RSD%
Estrone	0.72	3.65	2.90	5.90	10.37	1.62
17β- 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
17α-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 (± SD) of determined EDCs (ng/g, d. w.) in fish samples (n=3) collected from Llobregat river (Spain)

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

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

Compound	**Concentration range ± SD (ng/g, d.w.)**																
	Salmo trutta		***Gobio gobio***			***Pseudochondrostoma willkommii***	***Micropterus salmoides***			***Lepomis gibbosus***		***Aburnus alburnus***		***Anguilla anguilla***		***Barbus graellsii***	***Luciobarbus sclateri***
	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±6.58	<MDL	<MDL	<MDL	<MDL	4.45±0.44	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	2.97±0.13	<MDL	<MDL	<MDL
ylparaben	0.82 (A); 0.78 (J)	<MDL	<MDL	<MDL	<MQL	<MDL	<MDL	<MDL	<MDL	<MQL	<MDL	<MQL	<MDL	<MDL	<MQL	<MQL	<MDL
ylparaben	<MDL	7.43±0.69	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
zylparaben	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.54	0.38	<MDL	<MDL	0.33±0.01	0.50±0.04	<MDL	<MDL
eine	20.49 (A); 11.71 (J)	21.40±0.95	2.75	9.95 (A); 16.20 (J)	1.91	12.91	9.27±0.54	10.55	<MQL	5.83	1.15	<MDL	1.93	9.86±1.19	3.54±0.45	4.35±0.78	2.36±0.36
P	<MDL	10.60±5.69	<MDL	<MDL	<MDL	<MDL	7.79±5.97	<MDL	8.01±0.24	<MDL	<MDL	<MDL	<MDL	9.16±4.10	11.07±2.28	3.72±1.08	9.88±3.21

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

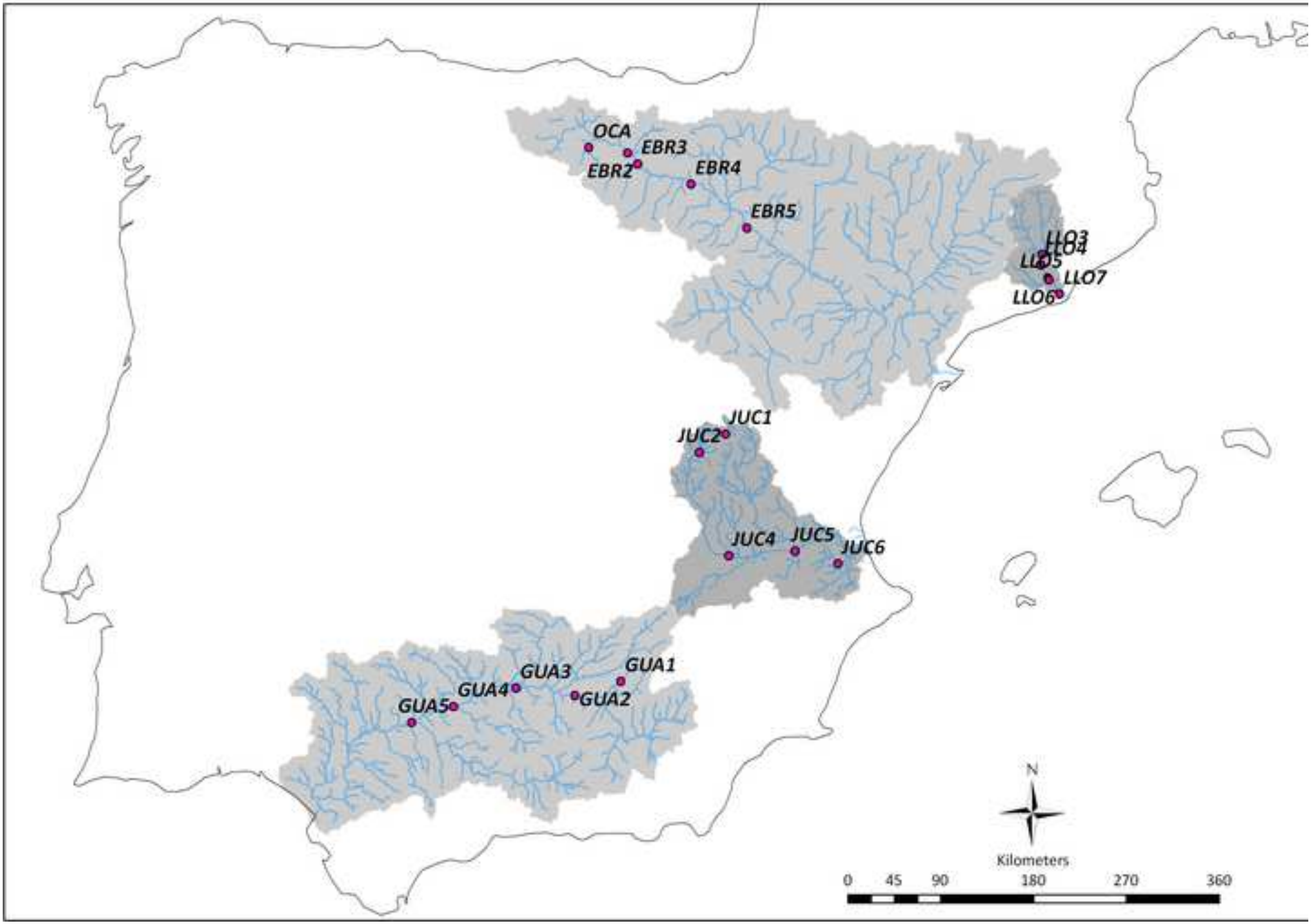
Compound	Concentration range ± SD (ng/g, d.w.)										
	Barbus graellsii					*Cyprinus carpio*				*Silurus glanis*	
	Ebro River – Sampling point										
	OCA	EBR2	EBR3	EBR4	EBR5	EBR2	EBR3	EBR4	EBR5	EBR4	EBR5
Estrone	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	1.99±0.19	<MDL	<MDL	<MDL	<MDL
Methylparaben	<MDL	<MDL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

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

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

(A) – adult; (J) – juvenile

Figure 1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EBR3
EBR2
EBR4
EBR5
LLO3
LLO4
LLO5
LLO7
LLO6
JUC1
JUC2
JUC4
JUC5
JUC6
GUA1
GUA3
GUA4
GUA2
GUA5
N
Kilometers
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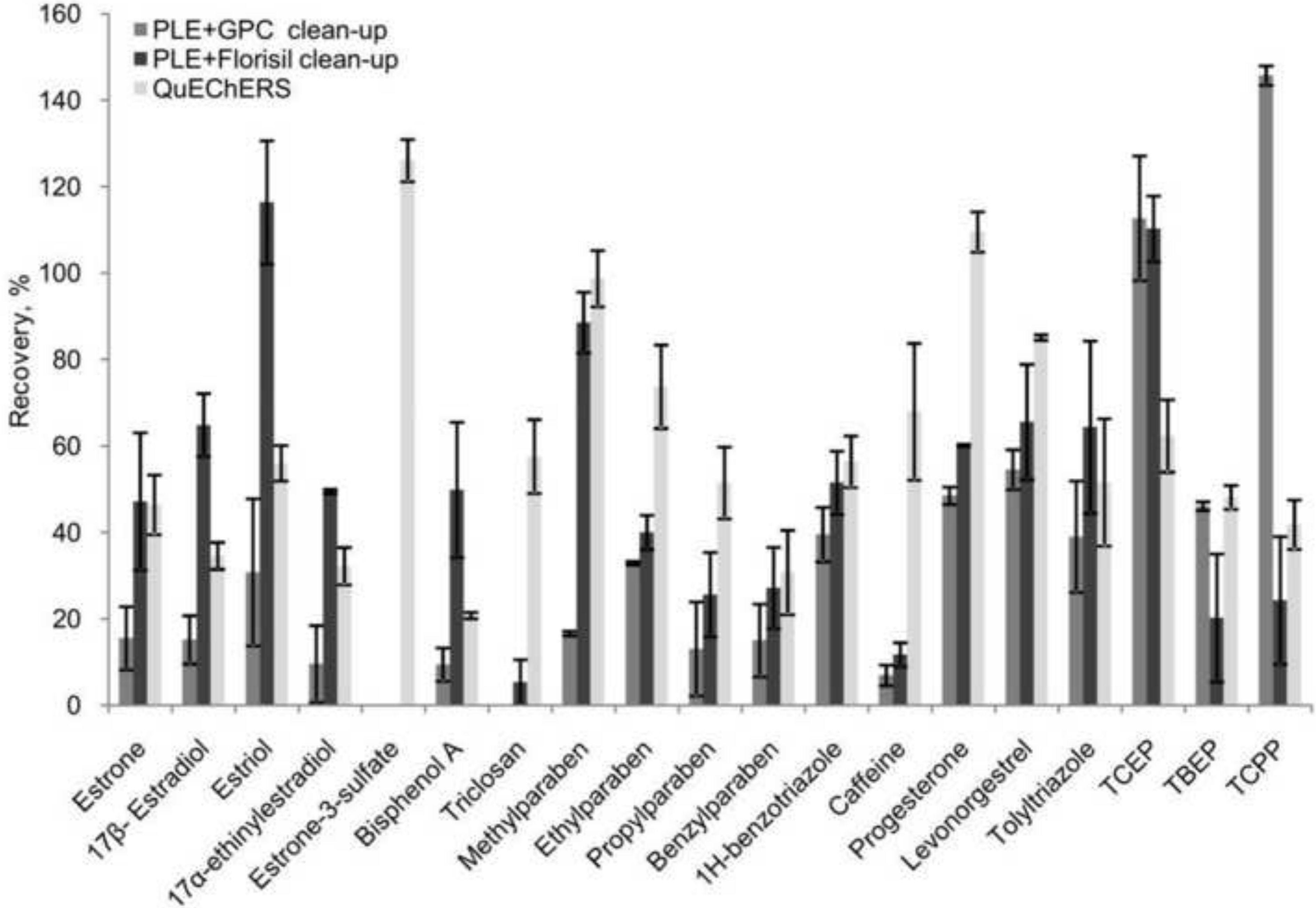
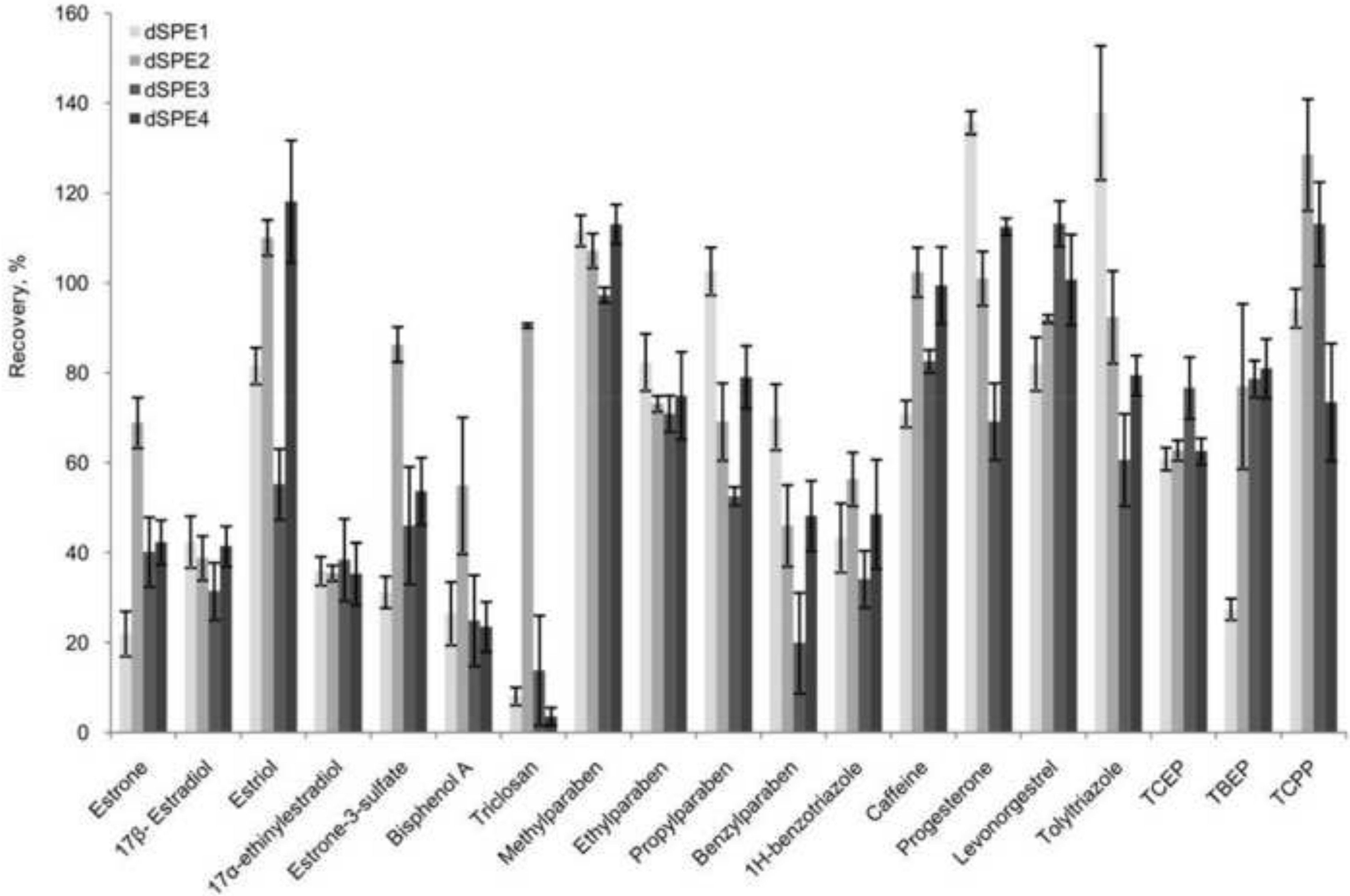
Figure 2



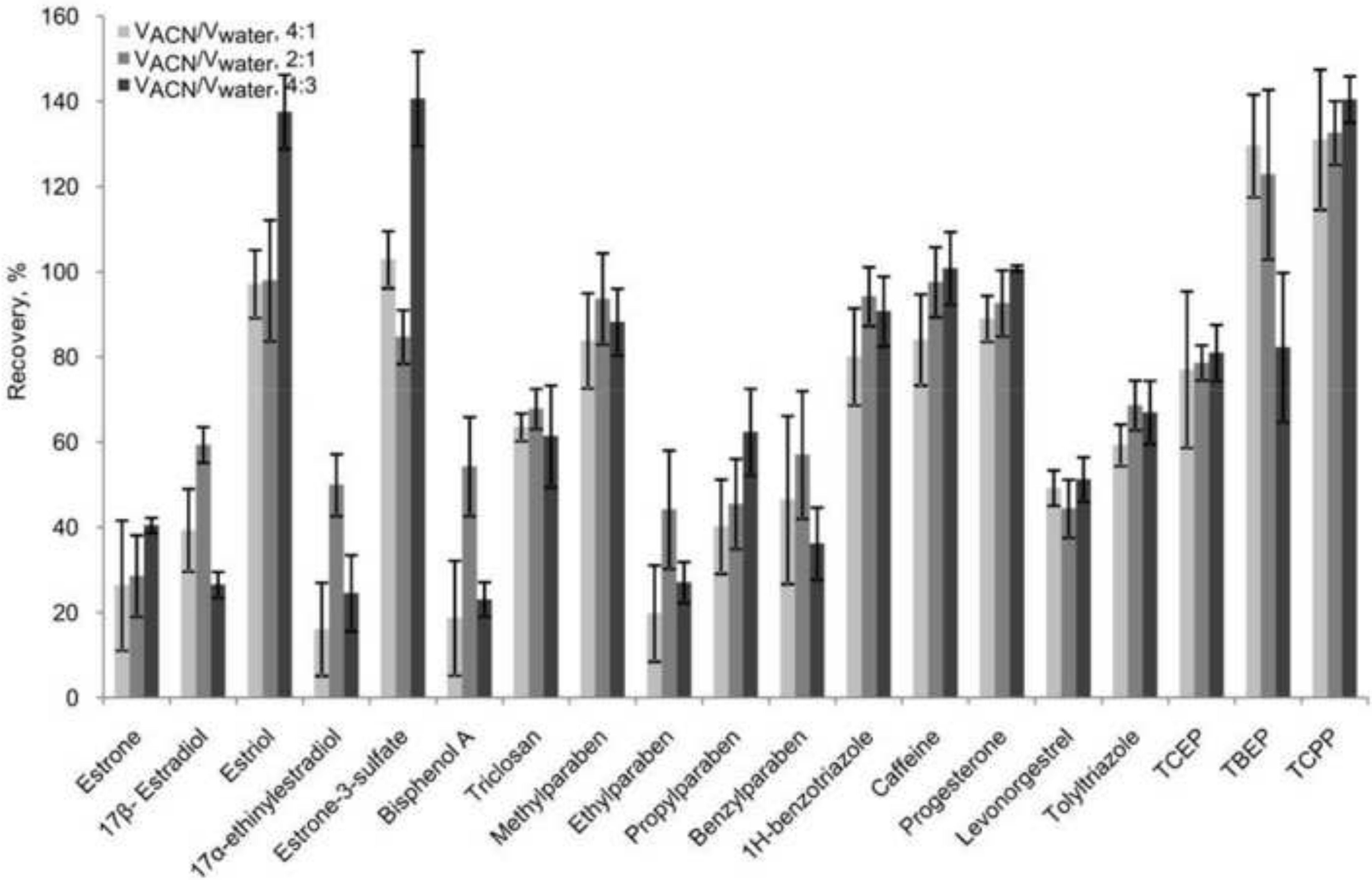
PLE+GPC clean-up
PLE+Florisil clean-up
QuEChERS
Recovery, %
160
140
120
100
80
60
40
20
0
Estrone
17β- Estradiol
Estriol
17α-ethinylestradiol
Estrone-3-sulfate
Bisphenol A
Triclosan
Methylparaben
Ethylparaben
Propylparaben
Benzylparaben
1H-benzotriazole
Caffeine
Progesterone
Levonorgestrel
Tolyltriazole
TCEP
TBEP
TCPP

Figure 3



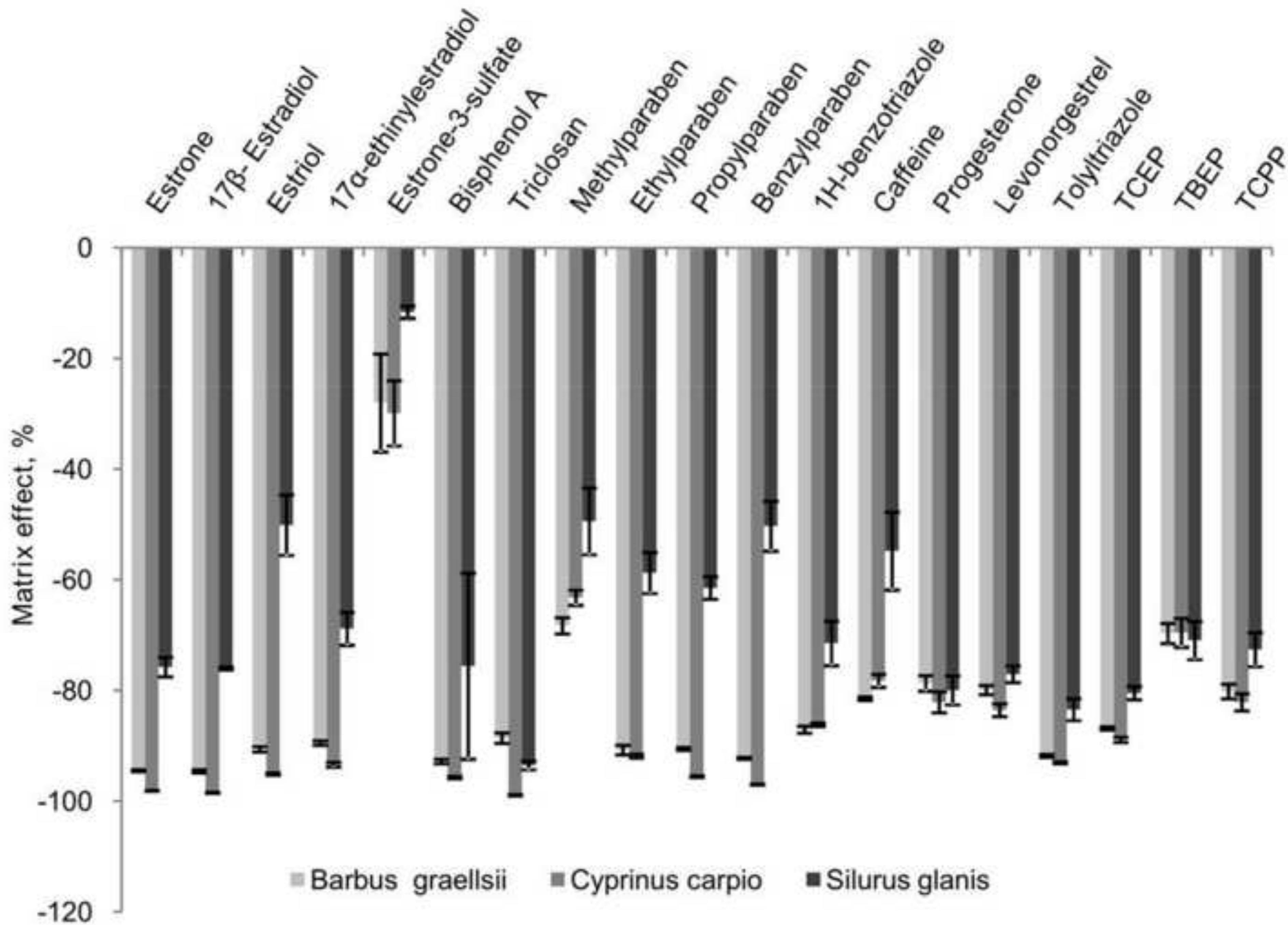
160
140
120
100
80
60
40
20
0
Recovery, %
dSPE1
dSPE2
dSPE3
dSPE4
Estrone
17β- Estradiol
Estriol
17α-ethinylestradiol
Estrone-3-sulfate
Bisphenol A
Triclosan
Methylparaben
Ethylparaben
Propylparaben
Benzylparaben
1H-benzotriazole
Caffeine
Progesterone
Levonorgestrel
Tolyltriazole
TCEP
TBEP
TCPP

Figure 4



160
140
120
100
80
60
40
20
0
Recovery, %
VACN/Vwater, 4:1
VACN/Vwater, 2:1
VACN/Vwater, 4:3
Estrone
17β- Estradiol
Estriol
17α-ethinylestradiol
Estrone-3-sulfate
Bisphenol A
Triclosan
Methylparaben
Ethylparaben
Propylparaben
Benzylparaben
1H-benzotriazole
Caffeine
Progesterone
Levonorgestrel
Tolyltriazole
TCEP
TBEP
TCPP

Figure 5


Estrone
17β- Estradiol
Estriol
17α-ethinylestradiol
Estrone-3-sulfate
Bisphenol A
Triclosan
Methylparaben
Ethylparaben
Propylparaben
Benzylparaben
1H-benzotriazole
Caffeine
Progesterone
Levonorgestrel
Tolyltriazole
TCEP
TBEP
TCPP
0
-20
-40
-60
-80
-100
-120
Matrix effect, %
Barbus graellsii
Cyprinus carpio
Silurus glanis