Monitoring pharmaceuticals in the aquatic environment using Enzyme-Linked Immunosorbent Assay (ELISA) – a practical overview

Guilaine Jaria[1], Vânia Calisto[1], Marta Otero[2], Valdemar I. Esteves[1]*

[1]Department of Chemistry and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Department of Environment and Planning and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

* Corresponding author: vania.calisto@ua.pt

Abstract

The presence of pharmaceuticals, which are considered as contaminants of emerging concern, in natural waters is currently recognized as a widespread problem. Monitoring these contaminants in the environment has been an important field of research since their presence can affect the ecosystems even at very low levels. Several analytical techniques have been developed to detect and quantify trace concentrations of these contaminants in the aquatic environment, namely high-performance liquid chromatography, gas chromatography and capillary electrophoresis, usually coupled to different types of detectors, which need to be complemented with time-consuming and costly sample cleaning and pre-concentration procedures. Generally, the enzyme-linked immunosorbent assay (ELISA), as other immunoassay methodologies, is mostly used in biological samples (most frequently urine and blood). However, during the last years, the number of studies referring the use of ELISA for the analysis of pharmaceuticals in complex environmental samples has been growing. Therefore, this work aims to present an overview of the application of ELISA for screening

and quantification of pharmaceuticals in the aquatic environment, namely in water samples and biological tissues. The experimental procedures together with the main advantages and limitations of the assay are addressed, as well as new incomes related with the application of molecular imprinted polymers to *mimic* antibodies in similar, but alternative, approaches.

Keywords

Emerging contaminants; organic micropollutants; ultra-sensitive analytical methods; antibodies; environment; biological tissues; environmental screening

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1. Introduction

Environmental pollution is an issue with multidisciplinary impacts and implications and water pollution is one of the most relevant problems of the current century. All living organisms depend on water and its contamination worsen even more the scarcity of potable water resources. A new global water quality threat is related to the so-called emerging contaminants, which are not commonly monitored but have the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects [1]. Pharmaceuticals are an important group amongst these emerging contaminants that have been under increasing scientific scrutiny [1-3]. In general, their occurrence in the environment ranges between the nanograms to micrograms per litre [1, 4, 5].

The presence of pharmaceuticals in aquatic systems is well-documented [6-15]. They have been detected in influents and effluents from Sewage Treatment Plants (STPs), groundwater, surface water, and even drinking water [10, 16-22]. The low concentrations of pharmaceuticals in the aquatic environment implies the need for techniques with adequate sensitivity for its monitoring. In this context, conventional chromatographic techniques are commonly used for this purpose, namely, gas chromatography (GC) and liquid chromatography (LC) [23-28], and several reviews on the subject appear in the scientific literature [15, 29-32]. Generally, these techniques involve coupling to expensive mass spectrometers detectors and complex sample pre-treatment procedures (purification, extraction, derivatization, and others), to achieve very low detection limits but resulting in more expensive and time-consuming analyses [32, 33]. Therefore, methods that simultaneously combine high sensitivity with simple, rapid and low-cost protocols are extremely important regarding periodic chemical analysis and large-scale environmental screenings [34]. Table 1 presents an overview of sensitive conventional analytical methods

(based on LC or GC) developed for monitoring pharmaceuticals in the aquatic environment. The presented examples highlight the main advantages of using methods based on chromatographic techniques, such as very low detection (LOD) and quantification limits (LOQ), and the ability to perform multi-analyte analyses [35-39]. However, these techniques also present some limiting factors such as sequential processing and the need to be coupled to powerful detectors and/or the use of extraction/clean-up methods for environmental samples (in order to enrich the sample concentration or to avoid matrix interferences) to achieve low LOD and LOQ. There are also reports of using more than one chromatographic technique (e.g. HPLC fractioning followed by GC analysis or more than one extraction technique) together with enrichment procedures in order to achieve the required LOD [40]. All this combined involve very high costs not only in what concerns the analysis itself, but also the specialized technicians needed to operate such instruments and analyse the resulting data [41].

Immunochemical techniques, which are based on specific antibody-antigen interactions, are intensively used in clinical analyses and have been considered as an alternative to conventional chromatographic techniques for monitoring trace amounts of several organic, an even inorganic, pollutants [42, 43]. The first application of ELISA for environmental purposes goes back to 1971 [44, 45]; afterwards, in the 1980s', ELISA was described as a potential technique to monitor agrochemical and environmental pollutants [46] and started to be more commonly applied, at that time, to the quantification of pesticides, which were already regulated pollutants [44, 47]. Since research concerning the presence of pharmaceuticals in the aquatic environment is posterior, the use of immunoassays for monitoring these compounds is rarer [23, 48]. According to Deng et al. [49], until the beginning of this century, only six papers were published concerning the

applicability of immunoassays for the detection of pharmaceuticals in environmental water systems, one of which using an enzyme-linked immunosorbent assay (ELISA) [50] and two using radioimmunoassays [51, 52]. Later, it was also reported the use of ELISA for the quantification of pharmaceuticals in tissues, in aquatic biota, in order to understand the bioavailability, bioaccumulation and/or effects of these pollutants [53]. Indeed, aquatic organisms can be used as indicators of pharmaceutical pollution in natural waters, and ELISA may be a rapid and cos-effective technique to support such studies [53, 54]. The main advantage of immunoassays, more specifically ELISA, is that they present high sensitivity and specificity, and generally require straightforward protocols together with inexpensive and simple sampling procedure [42]. Furthermore, ELISA can be used as a rapid screening method enabling testing a large number of samples at low cost and with little operator training [55]. Although the use of ELISA for samples is still unusual, it is a growing field that may lead to the establishment of an interesting alternative to detect and quantify pharmaceuticals in the aquatic environment.

The use of ELISA to determine pharmaceuticals in the aquatic environment has thrived during the last decade, as the environmental occurrence of pharmaceuticals received significant attention in this period. Therefore, this manuscript aims to review the literature on the determination of pharmaceuticals in complex environmental water samples and biological tissues from aquatic biota, using ELISA as detection and/or quantification method.

2. Fundamentals of ELISA

Immunoassays are based on the specific interactions between an antibody and an antigen (the analyte). Additionally, tracers (compounds that are analogue to the antibody or

the antigen, which might be radioisotopes, fluorophores, chemiluminophores or enzymes), are used to detect the compound of interest [22, 64]. In the case of ELISA, this detection relies in the use of an enzyme-labelled antibody or enzyme-labelled antigen that functions as tracer or conjugate [22]. In ELISA, one of the reagents is bound to a solid phase, generally a 96-well microtiter plate, which allows the analysis of several samples at one time [64] (Figure 1).

2.1 Calibration curve and precision profile

Two of the most commonly adopted classifications of ELISA are the competitive and non-competitive assays. In competitive ELISA there is a competition between the enzyme-labelled antigen and the antigen present in the samples for a fixed and insufficient quantity of immobilized antibody (direct format). A substrate is added and interacts with the enzyme-labelled antigen that was retained in the immobilized antibody. The conversion of substrate to a product that can be measured, continuously in a kinetic assay, or at a fixed-time approach, after a given incubation time. This last method is the most used, stopping the reaction with a strong acid or base, which denatures the enzyme. The product concentration is inversely proportional to the analyte concentration (calibration curve in Figure 1).

Depending on the ELISA format, more specifically if it is a competitive or a non-competitive ELISA, different plots are used to determine the accuracy of the method. In competitive ELISA a four-parametric logistic equation (4PL) (equation 1) is used to determine the quantification range of the assay [22, 66].

$$y = \left[\frac{A-D}{1+\frac{x}{C}^{B}}\right] + D \text{ (equation 1),}$$

where *y* is the optical density (OD); *x,* the antigen concentration; *A*, the optical density (OD) for an infinitely small analyte concentration ("blank"); *B*, the slope at the inflection point; *C*, the concentration value at the inflection point; and *D*, the OD for an infinite analyte concentration (standard excess) [22, 65]. The inflection point concentration (*C*) corresponds to the test midpoint of the ELISA calibration curves when results are fitted to a four-parameter logistic function and is similar but not equal to half of the maximum inhibitory concentration (IC_{50}) [66].

Another important procedure in the validation of ELISA is the setup of the precision profile. The precision profile is useful to establish the quantitation or working range of the assay, for a given level of precision, for which the method can be used [22, 65, 67]. It indicates the quality of the assay and is defined through the relative errors of the analyte concentrations (or coefficient of variation, CV) which are calculated from the OD standard deviations and the slope (1st derivative) at each individual standard concentration (equation 2) [22, 65].

$$CV\ (\%) = \frac{\sigma_y}{slope \times x} \times 100\% \quad \text{(equation 2)},$$

where σ_y is the standard deviation of the response of the replicates [22, 65]. The precision profile is represented graphically by plotting the random error in the analyte measurement for each value of analyte concentration (Figure 1).

2.2 Advantages and limitations

The application of ELISA to monitor environmental samples started in the 1980s [46]; still, its use in this context is more recent than other techniques, such as HPLC, GC or CE, and has not been so intensively explored as the later. However, some published papers

show that this technique has potential for environmental applications and can present some advantages relatively to the traditional chromatographic techniques.

The main advantages shown by ELISA are the low detection limits without sample pre-treatment, the analysis of several samples simultaneously, and the low cost of the equipment. ELISA is also a good tool to assess the spatial and temporal distribution of a single analyte [41] or to be used in a first approach in large-scale environmental screenings.

The main limitations of ELISA include the use of antibodies, which sometimes could be expensive; the possible need of coupling sample pre-treatment procedures; cross-reactivity (CR) effects due to the presence of compounds similar in structure to the target analyte; and, as for other analytical techniques, matrix effects (mainly, pH, natural organic matter, and salinity) [67]. Also, the use of organic solvents can be problematic due to enzyme denaturation. Also, ELISA is known to be mostly a single analyte technique, which is a disadvantage relatively to other chromatographic techniques that possess multiplex capacities. To overcome this, there have been an increase trend in the development of multiplex immunoassays using antibody microarrays chips and bead-based assays [68, 69]. Studies on the application of multiplexed assays for the determination of pharmaceuticals in real aquatic samples are scarce. An interesting example is the work by Carl et al. [7[]0] who successfully applied a multiplexed ELISA for the determination of caffeine, carbamazepine, diclofenac and isolithocholic acid in real wastewater samples, with subsequent validation with LC/MS/MS. However, these techniques also present their own disadvantages mainly related with CR that tend to increase with increasing number of target analytes [68]. In fact, the referred limitations of ELISA, CR is one of the most relevant in what concerns environmental samples, being an indicator of the assay specificity. CR is related to the response of the antibody when in presence of other compounds usually

similar to the analyte [67]. CR is defined as the mass or concentration of interferent required to displace 50% of the label. So, the percentage of CR is equal to 100 times the concentration of analyte (S) at 50% response divided by the concentration of interferent (I) at 50% response (equation 3).

$$\%CR = \frac{[S]_{50}}{[I]_{50}} \times 100 \quad \text{(equation 3)}$$

The presence of structurally similar compounds presenting significant CR can influence the specificity of the immunoassay and overestimate the concentrations of the target analyte or even generate false positives. For that reason, it is important to assess the specificity of the antibody and the evaluation of possible cross-reactants present in samples in order to adequately use ELISA, as a quantification/screening method, for environmental monitoring. Yet, the selection of possible cross-reactants might be very challenging. Cross-reactants with unexpectedly different chemical structure can appear, especially when the matrices are as complex as environmental samples.

3. ELISA as a tool for monitoring pharmaceuticals in the aquatic environment

In the past 20 years, a substantial amount of work has been done to determine the occurrence, fate, effects, and risks of pharmaceuticals in the environment [71]. In that sense, since ELISA has been applied to monitor the presence of these organic contaminants in water systems and also in biological tissues, some of them from organisms used as indicators of environmental pollution [22, 23, 41, 49, 66, 72-77], the following two sections present the literature related to this subject.

3.1. Pharmaceuticals monitoring in water samples

ELISA has been used alone as an analytical tool to detect and/or quantify pharmaceuticals in the environment, however there are also some reports about the use of ELISA combined with extraction and preconcentration methods to enhance the detection signal or to clean-up the samples. Tables 2 and 3 present a summary of relevant studies on the application of ELISA to the detection of pharmaceuticals, namely non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics and central nervous system (CNS) stimulants (presented in Table 2) and endocrine disruptors and hormones (presented in Table 3), in environmental aqueous matrices. Those studies are discussed below in detail.

3.1.1. Direct determination of pharmaceuticals in untreated samples

One of the most commonly found pharmaceuticals in surface waters is carbamazepine (CBZ). CBZ has been proposed as a marker of anthropogenic pollution [66, 77] and the screening of this pharmaceutical in environmental samples through ELISA has been studied by some authors [41, 66, 79] (Table 2). The sensitivities of the assays used for the detection of CBZ are in the order of the low micrograms per litre, the lowest being the quantification limit achieved by Bahlmann et al. [41] (~0.025 $\mu g\ L^{-1}$), all obtained without sample preconcentration. In these studies, CR effects, pH, ionic strength, and matrix effects were analysed in order to optimize the method and the validation was performed using LC-MS/MS as reference method. Indeed, the validation of ELISA by a reference technique has allowed for the detection of overestimation due to matrix or CR effects. In a work by Bahlmann et al. [79], it was considered that two CBZ metabolites - epoxycarbamazepine (EP-CBZ) and 2-hydroxycarbamazepine (2OH-CBZ), with molar CRs of 83% and 14%, respectively - were the main responsible for the overestimation of the ELISA results, since they were probable to be found in the environment (CBZ can be excreted as 3% EP-CBZ

and 5-6% 2OH-CBZ). Interestingly, the metabolite 10,11-dihydro-10,11-dihydroxy-CBZ (DiOH-CBZ) is excreted by humans at higher rates than the parent compound itself and, thus commonly occurs in the environment often at higher concentrations than CBZ [41]. However, this metabolite is not a relevant cross-reactant of this ELISA and in this sense hardly influences the CBZ determination [79]. A few years later, the same authors carried out an in-deep and comprehensive analysis on the role of CBZ metabolites in the determination of this pharmaceutical by ELISA. For that purpose, Bahlmann et al. [96], recurring to LC-ELISA, concluded that a 30% overestimation of the ELISA results for CBZ was not only due to the CR of the previously referred metabolites, seeing that the concentration that they presented in the samples were too low for such an overestimation. Surprisingly, the observed 30% overestimation in the concentration of CBZ in water samples, was mainly due to the cross-reaction of cetirizine (CET), an anti-histaminic drug, which does not have structural similarities with CBZ. The identification of this cross-reactant was possible through LC fractionation of the sample before ELISA analysis followed by LC-MS/MS analysis. The authors performed an investigation of the CRs of CET and its derivatives/metabolites, namely norchlorcyclizine, that indicated that these two compounds showed high CR, especially at low pH values (4.5), probably due to the protonation of the amine group in a distance of four atoms from the azepine's nitrogen. Also, the crystal structures of CBZ and norchlorcyclizine were analyzed (since, at that time, the CET crystal structure was not known) allowing to conclude that the antibody possessed a "blind spot" on the atomic junction between the CBZ phenyl rings, which was the main cause of the CR for other compounds such as CET and 10,11-dihydro-10,11-epoxycarbamazepine [96]. In the same work, it was shown that pH also influenced the enantio-selectivity, where antibody's affinity showed to be higher for (S)-CET than for (R)-

CET increasing from 4 times to more than 30 times from pH 4.5 to 10.5. This study revealed that the high environmental concentrations of the anti-histaminic pharmaceutical CET clearly justified the impact of this pharmaceutical in the CBZ determination using this assay; the highest overestimations were found in the spring and summer due to the seasonality of CET prescription [96].

This pH feature of the above-mentioned assay was used by Calisto et al. [66] to simultaneously determine the concentration of CBZ and CET (the main cross-reactant). As previously referred, the selectivity of the monoclonal antibody towards CET and CBZ was proven to be highly dependent on pH. Hence, two pH values, 4.5 (maximum CET selectivity) and 10.5 (maximum CBZ selectivity), were selected to study the CR and the concentrations of both pharmaceuticals using a system of equations considering the CR and the assay signal at both pH [66]. An overestimation of the concentration of CBZ (about 2-29%) was still observed when comparing with the LC-MS/MS results, possibly due to the cumulative contributions of matrix effects with the presence of the identified CBZ metabolites, EP-CBZ or 2OH-CBZ, also recognized by the used antibody. These two examples [66, 96] show how CR effects, usually considered a disadvantage, might also open the possibility of using ELISA for the determination of more than one pharmaceutical by just changing the pH of the assay. However, the application of multi-analyte ELISA for the specific and individual quantification of different pharmaceuticals in the aquatic environment is scarce. In a different way, multi-analyte ELISA has been used for the recognition and quantification of pharmaceutical families. This is the case of Adrian et al. [97] who performed a multi-analyte detection for three families of antibiotics (sulfonamides, fluoroquinolones and ß-lactams) in milk samples. On the other hand, multi-analyte ELISA has been also applied for the quantification of some organic contaminants in

the aquatic environment, such as pesticides [98-101], which are out of the scope of this review.

Caffeine (CAF) is another compound that, along with CBZ, has been proposed as anthropogenic marker for wastewater contamination of surface waters. In Table 2, three studies [41, 78, 74] are mentioned concerning the use of ELISA for the quantification of CAF in environmental samples. The study of Bahlmann et al. [41] is particularly interesting as it applies ELISA to determine CAF, CBZ and CET in environmental samples in order to assess their spatial and temporal variation in surface and wastewaters. This study showed the capabilities of ELISA to detect the referred compounds during extensive water sampling campaigns, where the application of conventional techniques would be extremely time-consuming. It was observed that several factors influenced the fluctuations of the concentrations of the pharmaceuticals, for instance, the input of STPs, the variations on the flow rates of the STPs effluents and/or the incomplete mixing of the effluent with surface waters, and the oscillations of the fresh surface water levels during different seasons. Moreover, in the case of CET, it was verified that concentration variations were also correlated with the seasonal occurrence of pollen (that increases the consumption of this pharmaceutical). Similarly to Calisto et al. [66], the detection of CBZ and CET was made simultaneously taking the advantage of an antibody's pH-dependent selectivity. Samples were also analysed by LC-MS/MS, with or without SPE enrichment, in order to validate the results. Overall, the ELISA method proved to be sensitive and accurate, mainly for CBZ and CET, since the results of CAF were systematically overestimated when compared with the ones obtained by LC-MS/MS, mainly for effluent wastewater and surface waters [41]. The interference of the CAF metabolites xanthine and dimethylxanthine was excluded by LC-ELISA and this overestimation was attributed to a possible cross-reactant that was not

identified in the cited work. This work also highlights the spatial variations in the concentrations that, mainly in the case of CAF and CBZ, allowed to perceive a correlation between the concentrations of these pharmaceuticals and to identify points of contamination [41].

Sulfamethoxazole (SMX) is a bacteriostatic antibiotic belonging to the sulfonamides family, and together with sulfapyridine and their acetyl metabolites, it is amongst the most frequently detected compounds in the aquatic environment, sometimes with a prevalence of 100% in wastewater effluents [22, 83]. Generally, methods such as LC and CE coupled with amperometric or mass detectors, or even UV detector but with a previous step of off- or in-line pre-concentration, are needed to achieve detection/quantification in aquatic environmental samples [22, 83]. To overcome that, Silva [22] developed a sensitive method based on direct competitive ELISA to quantify SMX in environmental water samples (Table 2). The developed ELISA method showed an overestimation of the results when compared with LC-MS/MS attributed to probable CR effects. In that sense, the CR by similar compounds to SMX, namely other sulfonamides, was studied by Hoffmann et al. [83] which developed an ELISA method with a pre-step of LC fractionation (LC-ELISA) to quantify SMX in water samples (Table 2). The application of a LC fractionation prior to the ELISA measurements had also been applied by Bahlmann et al. [96] in order to identify cross-reactants of CBZ, as mentioned earlier in this section. However, in the case of Hoffmann et al. [83], the application of the LC fractionation was intended to overcome general matrix effects. With this LC-ELISA, which allowed for a 1000-fold enrichment of the samples, a quantification limit of 1 ng L^{-1} was theoretically possible, considering no matrix interference. The effects of CR of 24 similar compounds was investigated, as well as other matrix effects. It was disclosed that succinimidyl-

sulfamethoxazole (SMX-Succ), N-acetyl-SMX and sulfamethizole were the most problematic compounds with high CR, the last one presenting a higher antibody affinity than SMX itself. The high affinity of the antibody for these three compounds is related with the similarity of their spatial structure and the high electron density in the aromatic ring of the molecule. Another interesting aspect has to do with the fact that cross-reactant compounds with relevant structural similarities can present low CRs as a consequence of having their structure sterically hindered, as it was the case of SMX-β-D-glucuronide. The authors concluded that the fractionation of the environmental samples before the ELISA allowed to eliminate most of the interferences. This LC-ELISA was validated by LC-MS/MS and despite the good agreement between the methods, LC-ELISA presented some disadvantages such as the need of pre-concentration and LC fractionation steps, which eliminate the operational simplicity typical of ELISA, and high CR for similar compounds that occur in environmental water samples [83].

Diclofenac is a therapeutic agent that has been proposed as a priority hazardous substance [2, 102]. Among the most sensitive methods used for the quantification of diclofenac is a GC-MS method with a LOQ of 6 pg L^{-1}, yet involving a relatively laborious sample pre-treatment and a derivatization step [103]. Other relevant methods were reported, namely, a SPE-UPLC-QqQMS/MS method with a LOD of 0.1 pg L^{-1} and LOQ of 0.2 ng L^{-1} described by [104], a SPE-GC-MS/MS with derivatization, presenting a LOD of 5 ng L^{-1} [105] and a SPE-HPLC-MS method with a LOD of 0.3 ng L^{-1} [106]. In what concerns ELISA, only two studies report the development of ELISA for the quantification of diclofenac (Table 2). Deng et al. [49], developed anti-diclofenac antibodies and a corresponding ELISA to detect/quantify this pharmaceutical in tap water, surface water, and wastewaters. The ELISA was based on an indirect competitive format presenting, in

pure water, a LOD of 6 ng L^{-1}, achieved with any sample preparation which, in this context, is a very interesting result when compared with the LOD and LOQ values achieved by conventional methods (Table 2). In this work, the metabolite 5-hydroxydiclofenac presented 100% of CR for the assay, due to its dichlorophenyl ring, while the other tested metabolites showed CRs below 2% [49]. After verifying an overestimation of the results in wastewater samples, and in order to disclose the influence of diclofenac glucuronide (a product of the metabolization of diclofenac in the human body), the authors applied enzymatic or acidic treatments to transform the conjugate into the original unconjugated form. With this experiment, and the simultaneous analysis of both treated and untreated samples by ELISA and GC-MS, it was found that the probable cause of the overestimation could, in fact, be attributed to the presence of diclofenac glucuronide, not due to the high concentration of this metabolite in the environment but to the higher affinity of the antibody for the conjugate when compared with the parent drug [49]. While some authors refer that glucuronide conjugates are not prone to be probable CRs (as the structure of the parent compound is sterically hindered [83]), the opposite trend was suggested in this study [49]. The authors also referred an important obstacle in what concerns the evaluation of metabolites as cross-reactants: in most cases, the metabolites are not commercially available [49]. This also has direct implications on the environmental quantification of metabolites, which would allow to shed some light on the different forms in which the original drug can occur and the fate of those derivatives.

Another study by Huebner et al. [80] developed an ELISA based on a monoclonal antibody for diclofenac. The method proved to be reliable, without the need of a pre-concentration step, and stable to potential matrix interferences, namely, pH, calcium chloride concentration and humic acids (HA). It presented CR up to 10% for diclofenac

metabolites, but under 1% for other NSAIDs. The results compared very favourably with the reference technique SPE-LC-MS, presenting only differences of about 12% and 3% for wastewater and surface water, respectively, with the results of ELISA being slightly higher (Table 2).

The presence of indomethacin, a NSAID, in water samples was studied by Huo et al. [81]. The method developed by Huo et al. [81] was an indirect competitive ELISA presenting a LOD 500 times lower than the validation technique SPE-HPLC-UVIS, which needed a pre-concentration procedure by 100-fold. The correlation coefficient between ELISA and HPLC was 0.988, however, the ELISA presented an CR of 92.3% for acemetacin. The results obtained by ELISA (Table 2) were similar to the ones obtained with SPE-HPLC, however, due to the highest LOD, this last method was not capable of detecting indomethacin in one river sample. For other samples, the authors obtained an overestimation of the concentrations determined by ELISA (around 30%, in average).

Steroid hormones such as 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are amongst the most commonly estrogens found in wastewater [55] which belongs to a class of compounds that may interfere with the normal function of the endocrine system of humans and wildlife. As for the other contaminants addressed in this review, chromatographic methods are generally used to the detection of these compounds in environmental samples [107]. However, as compared with other pharmaceuticals, it is interesting to note the relatively large number of publications referring the use of ELISA as a screening method for estrogens (Table 3). Between the publications where ELISA is used to detect/quantify hormones, at least seven describe the use of commercial kits [84-86, 88-91] (Table 3). These kits present LODs in the range of ng L^{-1} to μg L^{-1}, however, most of them present high CR for similar compounds and are affected by the matrix, being,

therefore, more useful as screening tool than as quantitative methods. Indeed, ELISA kits, generally and when not coupled to sample treatment procedures, present higher LODs or LOQs than specifically developed ELISA methods.

In what concerns the development of ELISA and respective application in environmental samples for the detection of E2 and EE2, some interesting works have been published with particular focus on the enhancement of selectivity and sensitivity of the assay. For instance, Schneider et al. [92] used a chemiluminescence ELISA (CLEIA) to analyse EE2 in surface water and wastewater from STP effluents at sub-ppt levels (Table 3). The validation of the method was performed by LC-MS/MS, which involved a pre-concentration step by SPE to achieve a LOD as low as the one of ELISA. The results between CLEIA and LC-MS/MS were consistent showing the applicability of the method. Silva et al. [55] also studied the presence of estrogens in environmental samples (complex aqueous matrices) quantifying E2 and EE2 in surface and wastewaters using an ELISA without any sample clean-up procedures (Table 3). In this study, organic matter, represented by HA, was revealed to interfere more in the quantification of E2 than of EE2. The authors considered that this interference was probably related to the denaturation of proteins and enzymes in the presence of HA, which can bind to the Ab and/or to the tracer (unspecific binding). These matrix effects were overcome using a BSA sample buffer that was added to the wells prior to the addition of the analyte. The method allowed to quantify E2 in two wastewater samples, after primary treatment and after biological treatment, respectively, and in a surface water sample (Table 3).

Some of these studies addressed the importance of metabolites as CRs of these steroid hormones. In general, the main metabolites of E2 and EE2 mentioned as presenting CR for the developed ELISA methods are sulphates and glucuronides derivates (conjugated

at ring position 3) [87, 92, 93], similarly to other examples previously mentioned in this review.

3.1.2. Combining ELISA with extraction and pre-concentration procedures

Few papers refer the use of extraction and pre-concentration methods applied to ELISA. References to extraction methods are most commonly found due to the complexity of the sample matrix, which can also imply a concentration factor of the analyte. Pre-concentration procedures are not extensively applied because ELISA is already a sensitive method and does not rely on these procedures to achieve very low detection limits. However, when needed, the use of organic solvents in such procedures interferes with ELISA, since they can affect the conformation of the antibody [108], implying an extra step associated to the evaluation of the effects of organic solvents on the assay sensitivity. An example of the use of pre-concentration and purification methods applied to ELISA is the one described in Huang and Sedlak [85] which investigated the use of commercially available ELISA for the quantification of estrogenic hormones, namely, E2 and EE2, in wastewater effluents from STPs and surface waters. The authors validated the obtained results by GC-MS/MS. Prior to analysis, the samples were subjected to two pre-treatment steps: SPE followed by an HPLC fractioning clean-up. The HPLC clean-up was justified as a method to remove interfering compounds in both methods, namely NOM, and implied the collection (and posterior analysis) of 5 fractions of each samples and the subtraction of the average of the background signal in the quantification of the hormones. For ELISA the concentration factors varied from 225 to 1500 for wastewater samples and from 750 to 2400 for surface waters. With these pre-treatment steps, the LOD obtained for ELISA for both hormones, estimated as two times the background signal, was ~0.1 ng L^{-1} for

wastewater effluents and 0.05 ng L^{-1} for surface waters (Table 3). The GC-MS/MS presented a LOD (*S/N=3*) of 0.2-0.4 ng L^{-1}, however, for this analysis a higher concentration factor was needed (14000 to 24000 for wastewater samples and from 32000 to 64000 for surface waters) and an additional step was performed related to the derivatization. Moreover, GC-MS/MS could only be applied to E2 as derivatization of EE2 yielded poor recoveries. Therefore, GC-MS/MS was only used as confirmatory analysis for one surface water sample and the result was 21% higher than the one of ELISA. The overestimation of the GC-MS/MS method is justified by the possibility of problems in quantifications at concentrations close to the LOD. Despite the time-consuming pre-treatment steps, it could be concluded that those were important to achieve low detection limits and less interference of the matrix. However, in the case of EE2, the pre-treatment step did not improve significantly the LOD of the method when comparing with the LOD obtained by Schneider et al. [92] without a pre-concentration step (Table 3).

Lima et al. [75] also analysed E2 and EE2 in potable, surface and wastewater samples by direct competitive ELISA after applying a dispersive liquid-liquid microextraction (DLLME). The DLLME procedure lead to overestimation of the results, however it proved to be reliable if both standard and samples were subjected to the same extraction procedure. Working ranges of 1.2-8000 ng L^{-1} for E2 and 0.22-1500 ng L^{-1} for EE2 were obtained, which implied the decrease of the lower LOQ for both E2 and EE2 for about 30 and 100 times, respectively, comparing with ELISA without DLLME pre-treatment. Despite the good results, no reference analytical technique was applied to confirm the quantification of the hormones in water samples. Also, and as referred above, for the particular case of EE2, the pre-treatment step did not improve the LOD of the

method when comparing with the one obtained by Schneider et al. [92] without pre-concentration step but with a chemiluminescent detection (Table 3).

Hintemann et al. [87] also applied an extraction/purification procedure to surface and waste waters samples to quantify the compounds E2 and EE2 through ELISA (Table 3). The procedure involved an adjustment of pH and the use of several organic solvents (methanol, hexane, and acetone); however, the optimization of the assay concerning the final percentage of methanol in the samples is not mentioned. This pre-treatment step, despite laborious, allowed to achieve very low detection limits, when compared with other ELISA methods. Nevertheless, no validation of the results was performed using another analytical technique.

Later, Uraipong et al. [95] also developed a highly specific polyclonal Ab for EE2 and a competitive ELISA for the detection of this compound in environmental water samples (Table 3). The work focused on the synthesis of EE2-haptens with the spacer arm attachment at the C3 hydroxyl group. The objective was to enhance the selectivity of the polyclonal antibody for this hormone over other steroid hormones such as estriol, estrone, estradiol dipropionate, progesterone, 17α-estradiol, medroxyprogesterone, and E2, as their main structural difference resides in C17 and C18 functional groups. The results for CR showed a high selectivity for EE2 and for mestranol (MeEE2) due to the antibody binding to the ring D of EE2. However, the assay was sensitive to matrix and organic solvents, namely, pH, concentration of NaCl and HA above 0.01 M, and the presence of ethanol and methanol above 10%. Furthermore, and most important, the fact that the assay presented a CR of 118% for MeEE2 implicates that it was not possible to rely on the values detected in water samples. In fact, the authors referred to the pair EE2/MeEE2 when presenting the detected values. Nevertheless, the CR for E2 was of 3.1% and for other steroidal sex

hormones and their derivatives was below 0.5%. The ELISA method was validated with GC-MS, presenting a good correlation; still, this validation was performed with ultra-pure water spiked samples and not in real samples.

It is noteworthy that, in a large amount of studies, ELISA is applied without being preceded by complex extraction and/or pre-concentration procedures, as it is perceptible, in particular, in Table 2 (note that for the quantification of hormones (Table 3), the use of extraction and pre-concentration is much more often applied). Most of the times, and even for wastewater and surface waters, the QR range of the assay is adequate without recurring to pre-concentration factors. In these complex matrices, the main concern usually lies in matrix effects that influence the recognition of the pharmaceutical by the antibody, namely dissolved organic matter and salinity. Apart from an initial filtration of the samples, which is thoroughly applied in literature studies (e.g. [41, 49, 66, 79, 92]), diluting the samples and/or applying a sample buffer are commonly used strategies to overcome significant interferences. Dilution is more frequently applied in wastewaters, particularly influent wastewaters. Huebner et al. [80], in the quantification of diclofenac in wastewater, diluted the samples by 20-fold due to the high concentration found for this pharmaceutical. Also in the quantification of diclofenac, Deng et al. [49], tested several dilution factors (from 10- to 200-fold) and concluded that a 10-fold dilution with ultrapure water was enough to eliminate matrix effects. Again, dilution is only a possibility as a way of solving matrix interferences, due to the low quantification limits typical of these assays. In what concerns the use of sample buffer, it is introduced in assays to level out complex matrix characteristics that can affect the binding between the antibody and the antigen, and it is added to all samples and calibrators [79]. Its composition may vary depending on specific applications. Yet, most commonly, it includes a buffering agent (such as citrate, glycin or

tris(hydroxymethyl) aminomethane (TRIS), depending on the optimal pH for the assay); high concentrations of salt (NaCl) and EDTA (applied to saline samples or to counter the ionic composition of the samples) and bovine serum albumin (BSA) (which is thought to inactivate contaminants that might cause the denaturation of the antibodies, such as dissolved organic matter) [79]. Several works followed this approach with satisfactory results when handling complex aqueous samples, namely in the quantification of CBZ and CET [41, 66], CAF [74], SMX [22], E2 [55, 75].

3.2 Pharmaceuticals analysis in biological tissues of aquatic organisms

The presence of contaminants in the aquatic environment can directly affect some aquatic species (metabolism, oxidative stress, etc.), possibly resulting in bioaccumulation in the organisms' tissues. In this context, the determination of trace levels of organic contaminants in biological tissues is extremely relevant to better understand the impact of such contamination. Some studies have been made on the utilization of ELISA for the analysis of pharmaceuticals in biological samples (Table 4). These studies are important since some aquatic species can be used as indicators of environmental contamination [109]. The analytical determination of pharmaceuticals in biological tissues of relatively small organisms is quite challenging, mainly due the size of the sample, which makes difficult the application of the most common pre-concentration techniques. Most of the studies in literature focus on the effect of the drugs CBZ, CET, and CAF in several aquatic species, namely, in mussels [110], clams [54, 111-116], and polychaetes [116, 117]. In these studies, the application of ELISA allowed the quantification of the pharmaceuticals in biological tissues from organisms subjected to environmentally relevant concentrations of pollutants. For instance, several authors [73, 118, 119] studied the presence of CBZ in

environmental samples, namely in coastal systems, by assessing the influence of this compound in biological organisms, namely, the clams *Venerupis decussata, Venerupis philippinarus,* and *Scrobicularia plana*. In the work of Almeida et al. [118] it is shown that a direct competitive ELISA is a powerful technique to monitor the presence of CBZ in clams. The method proved to be sensitive (LOQ = 0.024 μg L^{-1}) and no significant matrix effects occurred. The samples were obtained only by mechanically pulverizing the soft tissues of the frozen organisms with liquid nitrogen. The pulverized clam tissues were subsequently extracted with deionized water. No other sample treatment was applied. A similar study was performed by and Freitas et al. [73] and Freitas et al. [119] to analyze the combined effect of the presence of CBZ and pH to *Scrobicularia plana*. The quantification of CBZ in the bivalve tissues allowed to conclude that the exposure of the clam *Scrobicularia plana* to water contaminated with 0.3 μg L^{-1} of CBZ for 28 days resulted in a bioconcentration factor of 0.7 ± 0.1.

4. Mimicking of antibodies with Molecular Imprinted Polymers: a possible strategy to foster the use of ELISA for the environmental monitoring of pharmaceuticals?

The analytic application of ELISA, in environmental monitoring of pharmaceuticals, is limited by: (i) the difficult task of the production of specific antibodies, implying expensive and time-consuming research; and (ii) proper storage of the antibodies to maintain their activity. In fact, Smolinska-Kempisty et al. [122] referred that antibodies might be the Achilles' heel of ELISA. Concerning the production of specific antibodies, this is particularly challenging for low molecular weight compounds, as it is the case of pharmaceuticals, which are not able to directly induce an immune response in animals and, thus, antibody production. The production of antibodies for this type of compounds is

achieved by its conjugation (and sometimes only after chemical modification) with carrier proteins that, as a whole, act as the antigen. Thus, this extra step for low molecular weight compounds is critical in antibody production, with possible influence in the affinity and specificity of the resulting antibody [44]. Also, as seen in section 3.1, and still concerning the antibodies' specificity, one of the ELISA's biggest problems is the recognition of other compounds (CR), leading to the overestimation of the results. To overcome the referred limitations of traditional antibodies, mainly for small molecules, several strategies have been employed: a) use of computer-aided molecular modelling to assist the design of haptens that optimize the synthesis of specific antibodies; b) use of antibody-like binders (either synthetic or biological) such as nanobodies, recombinant antibodies, aptamers, and molecularly imprinted polymers (MIPs) [44]. Considering biological antibody-like binders, and in the case of aptamers, a large number of examples can be found in literature for the determination of pharmaceuticals, although most of them lack the application to environmental samples. This subject has been focus of several literature reviews [123-125], where it is highlighted that sensitive and specific detection of low molecular weight molecules by these binders is still a challenge in real-world samples.

In the particular case of MIPs, these have been considered synthetic analogues of biological antibody-antigen systems, operating as a *lock and key* mechanism, allowing a specific selectivity to the molecules that were templated during their production [126]. Antibody *mimic* by molecular imprinting and its use in binding assays was proposed twenty years ago [127] and, since then, some studies have pointed to the advantages of using MIPs as substitutes of antibodies in immunoassays [127, 128]. First, and perhaps the most important advantage, is the fact that they can be synthetized, which allows to have a broader range of specific molecules to bind to specific target compounds. Also, MIPs can

be produced for target small molecules, unlike antibodies, which are only easily produced for macromolecules. Secondly, MIPs have higher durability than antibodies regarding storage conditions, such as temperature. Finally, production of MIPs is a low cost approach when compared with the production of antibodies [129]. These advantages give MIPs a great potential as substitutes for antibodies in ELISA, although limitations, such as the low sensitivity and specificity when compared with the immunoassay-grade antisera, cannot be disregarded [128].

The synthesis of MIPs and their analytic application, including their utilization in the so designated biomimetic ELISA-like assays (BELISAs) or pseudo-ELISA, has been addressed by some authors, as referred in the reviews by Bedwell and Whitcombe [126] and Chen et al. [130]. Regarding the specific application of MIP-based ELISA, some studies may be found in the literature. For example, a MIP-based ELISA was used for the quantification of vancomycin in buffer and blood samples, which presented a linear range of quantification between 0.001 and 70 nM [126, 131]. Smolinska-Kempisty et al. [122] successfully compared the performance of nanoMIPs with antibodies for four small molecule targets, including L-thyroxine, a medication used to treat thyroid hormone deficiency. In this study, the nanoMIPs-based assay showed comparable sensitivities to ELISA using mono- or polyclonal antibodies [122].

The application of MIPs-based ELISA for the detection of pharmaceuticals in the food [132-134] and clinical areas [135-137] has been widely developed in the last decade. Although such application in environmental samples is still very incipient, this field is certainly worth exploring and will probably undergo great progress in the near future. Indeed, promissory results were already obtained by Wang et al. [138], who conceived a fast and direct competitive BELISA for the determination of estrone in environmental

water. These authors synthetized an imprinted film of controlled thickness to be used as artificial antibody and their method exhibited excellent performance, with recoveries ranging from 80 to 95%, in the quantitative determination of estrone in river and lake water samples. Despite the low LOD achieved by the BELISA (8.0 ± 0.2 μg L^{-1}), the method also showed high CRs for other five estrogenic compounds, namely, 17β-estradiol, estriol, diethylstilbestriol, and progesterone, showing that it was not very selective towards estrone. Nevertheless, no significant differences were present when comparing the analysis of spiked water samples by HPLC and BELISA. Altogether, it may be said that MIPs-based ELISA can be an interesting variation to be implemented for the monitoring of pharmaceuticals in the environment, presenting several advantages related with the stability of the assays and the possibility of having larger spectrum of small molecules that can be detected.

5. Conclusions

ELISA, which is frequently used in clinical analyses, has been considered as an alternative to conventional chromatography-based techniques for the environmental monitoring of pollutants. For this purpose, a main advantage of ELISA is being a cost-effective technique that enables rapid screening analysis but requires little operator training and simple equipment. In the specific case of pharmaceuticals monitorization, the simultaneous analysis of multiple samples in a short period of time and the detection of very low concentrations of analyte, either with or without sample pre-treatment have been highlighted as highly advantageous in the literature. ELISA may achieve pharmaceuticals' quantification ranges in the order of the low μg L^{-1} level without sample pre-treatment, and of ng L^{-1} after applying a pre-concentration factor, which is an important feature in what

concerns the detection of trace levels of this type of contaminants. However, the use of ELISA for the environmental monitorization of pharmaceuticals is still not as common as chromatography-based techniques and there are important features and limitations that need to be overcome, such as cross-reactivity (implying lack of specificity) and the development of multiplexing abilities. The need for the production of specific antibodies to the target pharmaceutical can be also considered a disadvantage in the use of ELISA for the analysis of environmental samples. However, this last limitation of ELISA can be overcome by the use of natural or synthetic antibody inspired solutions, of which MIPs are an interesting example constituting a new application that will probably develop into an important research area in the near future.

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Compliance with Ethical Standards

The authors declare no conflict of interest.

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Table 1. Examples of sensitive conventional analytical methods developed for monitoring pharmaceuticals in the aquatic environment.

Method	Pharmaceuticals	Sample pre-treatment	Type of Sample	Detection and Quantification Limits	Detection in real samples	Reference
Liquid chromatography						
HPLC-Q-Orbitrap-HRMS	B-blocker Analgesic Lipid regulator Antibiotic CNS stimulant Antiepileptic Nonsteroidal anti-inflammatory drug Antidepressant Anti-hypertensive Veterinary sedative	Filtration SPE	STP wastewater	QR: 1-100 ng L^{-1} LOQs: 10-1000 pg L^{-1}	LCD: 0.2 ng L^{-1} for Pravastatin HCD: 12,000 ng L^{-1} for Caffeine	[56]
LC-MS/MS	Antibiotic Antidepressant Antiseptic Expectorant/mucolytic agent CNS stimulant Antiepileptic Anti-infectives Anti-inflammatory Analgesic	Filtration SPE	Surface water (river)	MDL_{min}: 2.3 ng L^{-1} for Difloxacin MDL_{max}: 94.3 ng L^{-1} for Sulfamethizole	LCD: 6 ng L^{-1} for Caffeine HCD: 10,234 ng L^{-1} for Caffeine	[57]
µUHPLC-MS/MS	Analgesic Non-steroidal anti-inflammatory drug	Centrifugation Filtration Online-SPE	Tap/drinking water	LODs: 0.001 ng mL^{-1} (Indomethacin) to 0.5 ng mL^{-1} (Acetylsalicylic acid) LOQs: 0.005 ng mL^{-1} (Indomethacin) to 0.1 ng mL^{-1} (Acetylsalicylic acid)	—	[58]
			Surface water	LODs: 0.005 ng mL^{-1} (Indomethacin) to 0.5 ng mL^{-1} (Acetylsalicylic acid) LOQs: 0.01 ng mL^{-1} (Indomethacin) to 1 ng mL^{-1} (Acetylsalicylic acid)		
			STP wastewater	LODs: 0.01 ng mL^{-1} (Indomethacin) to 1 ng mL^{-1} (Acetylsalicylic acid)		

				LOQs: 0.025 ng mL^{-1} (Indomethacin) to 2.5 ng mL^{-1} (Acetylsalicylic acid)		
HPLC-DAD	Antibiotic (ciprofloxacin)	SALLE	Tap water Bottled water Hospital wastewater	QR: 0.1 – 100 µg L^{-1} LOD: 0.075 µg L^{-1} LOQ: 0.25 µg L^{-1}	< LOD	[59]
			STP wastewater		0.83 µg mL^{-1}	
Micellar liquid chromatography-PDA	Antibiotics CNS stimulant Non-steroidal anti-inflammatory drug Endocrine disruptors	SPE	River stream	LODs: 0.019 – 0.247 µg L^{-1} LOQs: 0.058 – 0.752 µg L^{-1}	0.109 ± 0.002 to 1.204 ± 0.034 µg L^{-1} for Caffeine 0.295 ± 0.010 µg L^{-1} for Ciprofloxacin 0.188 ± 0.007 µg L^{-1} for Tetracycline 0.463 ± 0.022 µg L^{-1} for Norfloxacin	[60]
			Potable (tap) water		0.071 ± 0.003 µg L^{-1} for Caffeine	
UHPLC-MS/MS	Multi-class pharmaceuticals	Online SPE	Ultrapure water Tap water Lake water Ground water	LODs: 0.00119 – 0.623 µg L^{-1} LOQs: 0.00475 – 2.49 µg L^{-1}	—	[61]
Gas chromatography						
MS/MS-MRM	CNS stimulant	SPE	—	LODs: 0.2 ng L^{-1}	—	[62]
MS	Non-steroidal anti-inflammatory drug Analgesic Antihyperlipidemic	Automated aqueous derivatization SPME	Surface water (river)	LODs: 0.06 – 1.24 ng L^{-1}	n.d. – 0.48 ng L^{-1} for Flufenamic acid n.d. – 15.28 ng L^{-1} for Naproxen n.d. – 7.63 ng L^{-1} for Tolfenamic acid	[63]

CNS – Central nervous System; FID – Flame ionization detection; GC – Gas chromatography; HCD - Highest value of concentration detected; HPLC – High performance liquid chromatography; HRMS – High resolution mass spectrometer; LCD – Lowest value of concentration detected and above the detection limit; LODs: Limits of detection; LOQs: Limits of quantification; MDL_{min} – Minimum value found in method detection limit; MDL_{max} – Maximum value found in method detection limit; MS – Mass Spectrometry; MS/MS - Tandem

mass spectrometry; MRM – multiple reaction monitoring; n.d. – non detectable; PPCPs – Pharmaceuticals and personal care products ; QR: Quantification Range; SPE – Solid phase extraction; SPME – Solid phase microextraction; STP – Sewage treatment plant; TQD – Triple Quadrupole; UAE – Ultrasound assisted extraction; UHPLC – Ultra high performance liquid chromatography; μUHPLC – Micro ultra-high performance liquid chromatography

Table 2. ELISA for analysis of pharmaceuticals (nonsteroidal anti-inflammatory drugs, antibiotics and central nervous system stimulants) in aquatic environmental samples.

Compound	ELISA format	Quantification and Detection limits; Working and Quantification Ranges	Type of Sample	Sample preparation	Occurrence in environmental samples	Tested interferences (matrix effects)	Interferences found	Validation technique (correlation with ELISA)	References
Caffeine (CAF)	Competitive ELISA kit	LOD: 0.135 ng mL^{-1} IC50: 2.21 to 2.73 ng mL^{-1} QR: 0.175 to 5 ng mL^{-1}	River waters	Filtration and SPE	<LOD 170 to 4280 ng L^{-1}	CR	CR of paraxanthine – 63% CR of theophylline – 5.2% CR of theobromine – 2.8%	SPE-LC-MS/MS R^2 : 0.935	[78]
			Wastewater		830 to 14200 ng L^{-1}				
	Direct competitive ELISA	LOQ ~0.025 µg L^{-1}	Effluent wastewaters	Filtration and storage at -4 ºC	0.7 and 1.5 µg L^{-1}	CR	—	(SPE) LC-MS/MS R^2: 0.994 $y = 1.05\ C_{VT} + 0.23$	[41]
			Influent wastewaters		150 to 450 µg L^{-1}				
			Surface waters		0.52 and 3 µg L^{-1}				
		QR: 0.1 - 100 µg L^{-1}.	Surface water from estuarine shallow lagoon	Filtration and storage at -4 ºC	<LOD 0.1 – 0.66 µg L^{-1}	Salinity Organic matter (HA)	HA (reduced when using BSA buffer)	(SPE) LC-MS/MS R^2: 0.9996 $y = (1.50 \pm 0.01)\ C_{VT} + (0.040 \pm 0.021)$	[74]
			Surface freshwater		<LOD 0.109 – 9 µg L^{-1}				
			Water from public potable fountains		<LOD 0.14 – 0.58 µg L^{-1}				
			Wastewater		<LOD 0.17 – 15 µg L^{-1}				
Carbamazepine (CBZ)	Direct competitive ELISA	QR: 0.05–50 µg L^{-1} LOD:0.024 µg L^{-1}	Surface waters	Filtration and storage at -4 ºC	0.55 – 3.2 µg L^{-1}	CR pH	CR of 10,11-Epoxycarbamazepine (EP-CBZ) – 83%	(SPE) LC-MS/MS R^2: 0.996	[79]

			STP wastewater		2.3 µg L^{-1}	Ionic strength	CR of 2-hydroxycarbamazepine (2OH-CBZ) – 14%	y = 1.29 C_{VT} + 0.01	
		QR: 0.03 -10 µg L^{-1}	Surface water	Filtration and storage at -20 ºC	0.11 ± 0.02 µg L^{-1}	CR with CET pH Salinity	CR (mitigated by the adjustment of the pH of the assay)	LC-MS/MS *R^2: 0.9385 *$y = 1.26\ C_{VT}$ -0.064)	[66]
			STP wastewater		0.5 - 0.7 µg L^{-1}	Organic matter Ionic strength[a]			
		LOQ ~0.025 µg L^{-1}	STP wastewater	Dilution of 50-fold in influent wastewaters	1.5 – 2.8 µg L^{-1} 5 µg L^{-1}	CR[a] pH Ionic strength[a]	CR of metabolite 10,11-dihydro-10,11-epoxy-carbamazepine; CR of CET (mitigated by the adjustment of the pH of the assay)	(SPE) LC-MS/MS R^2: 0.978 y = 1.07 C_{VT} + 0.00	[41]
			Surface waters (upstream STP)	—	~ 0.1 µg L^{-1}				
Cetirizine (CET)	Direct competitive ELISA	QR: 0.03 -10 µg L^{-1}	STP wastewater	Filtration and storage at -20 ºC	0.23-0.60 µg L^{-1}	CR[a] with CBZ pH Salinity Organic matter Ionic strength[a]	CR (mitigated by the adjustment of the pH of the assay)	—	[66]
		LOQ ~0.025 µg L^{-1}	STP wastewater	—	0.5 to 0.8 µg L^{-1}	CR[a] with CBZ pH Ionic strength[a]	CR (mitigated by the adjustment of the pH of the assay)	(SPE) LC-MS/MS R^2: 0.899 y = 1.09 C_{VT} + 0.00	[41]
Diclofenac (DCF)	Indirect competitive ELISA	QR: 20 - 400 ng L^{-1} LOD: 6 ng L^{-1} IC_{50}: 60 ng L^{-1}	Surface water	Diluted	15 - 19 ng L^{-1}	CR Humic acids Organic solvents: methanol, ethanol, and DMSO	CR: 5-hydroxydiclofenac (100%)	GC-MS	[49]
			Tap and surface water	Dilution and fortification	2 - 6 ng L^{-1}				
			Wastewater influent	Filtration and dilution (10 times)	3.76 ± 2.34 µg L^{-1} 2.00 ± 0.51 µg L^{-1}			GC-MS (without enzymatic or acidic treatment)	

			effluent					R^2: 0.49 (r = 0.70) y = 0.90 C_{VT} +0.37	
	ELISA	LOD: 7.8 ng L^{-1} IC_{50}: 44 ng L^{-1}	Surface water	Filtration	<LOD – 0.079 µg L^{-1} 0.031 ± 0.0011 µg L^{-1}	pH calcium ion concentration humic acid content CR	CR: 4'-OH-DCF (11%); 5-OH-DCF (13%); and DCF–acyl glucuronide (8.5%)	SPE-LC-MS R^2: 0.968 p (paired t test): 0.78	[80]
			Wastewater	Filtration and dilution	1.6 – 3.9 µg L^{-1} 2.5 ± 0.72 µg L^{-1}			R^2: 0.88 p (paired t test): 0.056	
Indomethacin	Indirect competitive ELISA	IC_{50}: 0.10 – 0.25 ng mL^{-1} LOD: 0.01 ng mL^{-1}	Tap water	—	Below LOD	CR	CR with acemetacin (92.3%)	SPE-HPLC R^2: 0.988 y = 0.802 C_{VT} − 0.037	[81]
			Drinking water	—	Below LOD				
			Surface water (rivers)	Filtration and dilution (if needed)	0.024 and 0.109 ng mL^{-1}				
			Surface water (Poll)		0.857 ng mL^{-1}				
			Influent/effluent wastewater from STP	Filtration, dilution pH adjustment	1.412 / 0.871 ng mL^{-1}				
			Hospital drainage		2.574 ng mL^{-1}				
Sulfamethoxazole (SMX)	ELISA	LOD (90% B/B_0[b]): 0.030 µg L^{-1}	STP wastewater (final effluent)	Centrifugation and filtration	1.1 – 3.0 µg L^{-1}	CR	CR: sulfamethoxypyridazine (175%); sulfachloropyridazine (142%); sulfamethoxine (61%); and sulfamethizole (10%)	(SPE)LC-MS/MS	[76]
			River		N.D. – 0.09 µg L^{-1}				
	ELISA kit	LOD: 0.015 µgL^{-1}	POCIS sampled surface water (river streams in small towns)	POCIS extraction	~ 5 – 201 ng of SMX equivalents / POCIS	CR	CR: sulfamethoxypyridazine (174.4%); sulfachloropyridazi	HPLC-MS/MS	[82]

			POCIS sampled surface water (rivers at major city)		<20 - 669 ng of SMX equivalents / POCIS		ne (141.7%); sulfadimethoxine (60.7%); sulfamethizole (10.2%); sulfapyridine (3.4%); and sulfasalazine (3.2%)		
			POCIS sampled STP waters (at major city)		359 – 8174 ng of SMX equivalents / POCIS				
	Direct competitive ELISA	QR: 0.1–30 µg L^{-1}	STP wastewater	Filtration and storage at -4 ºC	4.3 – 11 µg L^{-1}	Salinity Organic matter (HA, FA)	Salinity and HA (mitigated with the use of BSA sample buffer in the assay)	LC-MS/MS *R^2: 0.2828 *$y = 25\ C_{VT} + 5$ *R^2: 0.1212 *$y = -21\ C_{VT} + 0.5$	[22]
			Surface waters		0.095 – 0.9 µg L^{-1}				
	LC-ELISA	WR: 0.06 – 360 µg L^{-1} QR: 0.82 – 63 µg L^{-1}	Influent wastewater	LC fractioning step	24 – 202 ng L^{-1}	CR Salinity Organic matter % Methanol	CR with SMX-Succ (1000%), N-acetyl-SMX (620%) and sulfamethizole (270%)	LC-MS/MS R^2: 0.99 $y = 0.93\ C_{VT} + 0.25$	[83]
			Effluent wastewater					R^2: 0.96 $y = 0.94\ C_{VT} + 1.15$	
			Surface water					R^2: 0.95 $y = 0.98\ C_{VT} + 0.58$	
Sulfamethazine (SMZ)	ELISA (with a magnetic particle immunoassay format	LOD (90% B/B_0[b]): 0.03 µg L^{-1}	market-weight hog wastewater	Centrifugation and filtration	~ 7.0 µg L^{-1}	CR	CR: sulfamerazine (23%)	(SPE)LC-MS/MS	[76]
			Swine wastewater (Piglet)		~ 30 µg L^{-1}				

BSA - bovine serum albumin; CR – cross reactivity; HA – humic acids; QR – quantification range (allows a maximum relative error of 10%); WR – working range (allows a maximum relative error of 30%) ; LOD – Detection limit; IC_{50} – half maximum inhibitory concentration; SPE – solid-phase extraction; DLLME – Dispersive liquid-liquid microextraction; C_{VT} – concentration measured by the validation technique; C_{ELISA} – concentration measured by ELISA method; N.D. – non detectable; POCIS – polar organic compound integrated sampler

[a] Tested in the development of the method; the procedure is described elsewhere

[b] absorbance value with competitor/absorbance value without competitor [76]

* The correlation values were calculated based on the data values presented in the referred publication.

Table 3 ELISA for analysis of endocrine disruptors and hormones in aquatic environmental samples.

Compound	ELISA format	Quantification and Detection limits; Working and Quantification Ranges	Type of Sample	Sample preparation	Occurrence in environmental samples	Tested interferences (matrix effects)	Interferences found	Validation technique (correlation with ELISA)	References
Estrone (E1)	ELISA kit	LOD: 0.2 ng L^{-1} LOQ: 5 ng L^{-1}	Influent wastewater (STP)	SPE	13 – 351 ng L^{-1} mean: 84 ± 97 ng L^{-1}	—	—	GC/LC-MS GC/LC-MS/MS	[84]
			Effluent wastewater (STP)		3 – 78 ng L^{-1} mean: 23 ± 25 ng L^{-1}				
			Upstream river		2 – 10 ng L^{-1} mean: 5 ± 4 ng L^{-1}				
			Downstream river		1 – 32 ng L^{-1} mean: 8 ± 9 ng L^{-1}				
17β-estradiol (E2)	Commercial ELISA	LOD: 0.1 ng L^{-1}	STP wastewater	SPE HPLC clean-up	0.24 to 3.8 ng L^{-1}	Natural organic matter	Mitigated by the HPLC-clean up	GC-MS/MS (C_{VT} ~ 4.75 times higher than C_{ELISA}	[85]
		LOD: 0.05 ng L^{-1}	Surface waters		0.05 to 0.8 ng L^{-1}				
	ELISA Kits (noncompetitive)	LOD: 0.5 ng L^{-1}.	Rivers (upstream STPs)	SPE extraction	1.9 – 2.0 ng L^{-1}	—	—	—	[86]
			Rivers (downstream SPTs)		2.3 – 6.0 ng L^{-1}				
			Ponds		1.7 - 7.6 ng L^{-1}				
			STP influents		18.9 - 71.2 ng L^{-1}				
			STP effluents		6.5 – 53.1 ng L^{-1}				
	Direct competitive ELISA	LOD: 0.05 ng L^{-1} LOQ: 0.36 ng L^{-1} WR: 28 – 590 ngL^{-1}	STP effluent	Filtration SPE	3.1 – 51 ng L^{-1} (mean concentration: 11.7 ng L^{-1})	CR	CR: sulfate-3-estradiol (9%) and glucuronide-3-estradiol (25%)	—	[87]

		Surface water (river)	Purification of the extract on silica gel column	1.3 – 9.2 ng L^{-1} (mean concentration: 4 ng L^{-1})				
ELISA kits / developed ELISA (E2-JEC)	WR / LOQs: 0.05 – 1 µg L^{-1}	STP primary effluent	Filtration SPE	21.1 – 200.2 ng L^{-1} / 12.5 ng L^{-1}	Use of methanol or dichloromethane in the extraction procedure	Methanol caused overestimation of the results	LC-MS/MS R^2: 0.95 (CH_2Cl_2) $y = 1.49\ C_{VT}$ (CH_2Cl_2)	[88]
		STP aeration tank	—	4.4 – 31.6 ng L^{-1} / 2.1 ng L^{-1}				
		STP secondary effluent	—	5.3 – 75.9 ng L^{-1} / 2.6 ng L^{-1}				
ELISA kit	LOD: 0.05 – 1.0 µg L^{-1}	Wastewater	Filtration Acidification, SPE	0.57 – 1.73 ng L^{-1}	—	—	LC-MS/MS	[89]
ELISA kit	LOD: 2.5 ng L^{-1}	Well water	Filtration	< LOD	—	CR with estradiol (50%)	HPLC-MS/MS R^2 : 0.999 $y = 1.6271\ C_{VT}$ UPLC-Q-TOF-MS	[90]
		River water	Filtration	< LOD				
			Filtration SPE	< LOD				
		Wastewater	Filtration	~ 2.5 – ~ 20 ng L^{-1}				
			Filtration SPE	< 0.5 – ~ 20 ng L^{-1}				
ELISA kit	—	Wastewater	Filtration SPE	2 – 5 ng L^{-1} 18 ng L^{-1}	—	—	—	[91]
Direct competitive ELISA	WR: 0.6-10 µg L^{-1}	STP wastewater Surface water	—	0.035-0.068 µg L^{-1} 0.085 µg L^{-1}	Salinity Organic matter (HA)	HA (mitigated with the use of a BSA-based sample buffer) Salinity without interference	—	[55]

	Direct competitive ELISA	WR: 1.2-8000 ng L^{-1} LOD: 0.22 ng L^{-1}	Public fountains (potable water)	DLLME	<LOD-2.0 ng L^{-1}	Organic matter (HA)	Mitigated with the use of a BSA-based sample buffer	[75]
			Surface water		<LOD-34 ng L^{-1}		—	
			Wastewater		21 and 77 ng L^{-1}			
	ELISA kit	LOD: 1 ng L^{-1} LOQ: 5 ng L^{-1}	Influent wastewater (STP)	SPE	20 – 199 ng L^{-1} mean: 119 ± 83 ng L^{-1}	—	—	GC/LC-MS GC/LC-MS/MS [84]
			Effluent wastewater (STP)		4 – 107 ng L^{-1} mean: 20 ± 31 ng L^{-1}			
			Upstream river		1 – 28 ng L^{-1} mean: 10 ± 15 ng L^{-1}			
			Downstream river		2 – 66 ng L^{-1} mean: 10 ± 18 ng L^{-1}			
17α-ethinylestradiol (EE2)	Commercial ELISA	LOD: 0.1 ng L^{-1}	STP wastewater	SPE HPLC clean-up	<0.1 to 1.98 ng L^{-1}	Natural organic matter	Mitigated by the HPLC-clean up	GC-MS/MS [85]
		LOD: 0.05 ng L^{-1}	Surface waters		<0.05 to 0.07 ng L^{-1}			
	Chemiluminescence ELISA	QR: 0.8 - 100 ng L^{-1} LOD: 0.2 ± 0.1 ng L^{-1} LOQ: 1.4 ± 0.8 ng L^{-1}	Surface water (river samples)	Filtration and storage at 4 ºC (24 h max)	0.6 – 0.7 ng L^{-1} < LOD	CR	CR with estradiol (0.2%) CR with estrone (0.1%)	SPE-LC-MS/MS [92]
			Effluent STP		0.4 - 0.7 ng L^{-1}			
	Direct competitive ELISA	LOD: 0.01 ng L^{-1} LOQ: 0.12 ng L^{-1} WR: 0.07 – 2570 ng L^{-1}	STP effluent	Filtration SPE Purification of the extract on silica gel column	0.4 – 3.3 ng L^{-1} (mean concentration: 1.8 ng L^{-1})	CR	CR with (in [93]): EE2-3-glucuronide sodium salt (17%) EE2-3-sulphate sodium salt (37%)	[87]
			Surface water (river)		0.3 – 1.0 ng L^{-1} (mean concentration: 0.7 ng L^{-1})		—	

						EE2-6-carboxy-methyloxime (99%) EE2-BSA (immunogen) (435 000%)		
ELISA kits / developed ELISA (E2-JEC)	LOQs: 0.05 – 3 µg L^{-1}	STP primary effluent / aeration tank / secondary effluent	Filtration SPE	0.8 – 3.1 ng L^{-1} / < 0.2 ng L^{-1}	—	—	LC-MS/MS	[88]
Direct competitive ELISA	WR: 0.03-40 µg L^{-1}	STP wastewater Surface water	—	<LOD <LOD	Salinity Organic matter (HA)	HA (mitigated with the use of a BSA-based sample buffer)	—	[55]
Direct competitive ELISA	WR: 0.22-1500 ng L^{-1} LOD: 1.2 ng L^{-1}	Public fountains (potable water)	DLLME	<LOD-1.32 ng L^{-1}	Organic matter (HA)	Mitigated with the use of a BSA-based sample buffer	—	[75]
		Surface water		<LOD-24 ng L^{-1}				
		Wastewater		6 and 8.5 ng L^{-1}				
Direct competitive ELISA [93]	LOD: 14 ng L^{-1} [93]	River (Grab samples)	SPE	< 0.4 ng L^{-1}	—	—	—	[94]
		River (POCIS samples)	—	N.D. 0.48 – 1.4 ng / POCIS				
ELISA kit	LOD: 5 ng L^{-1} LOQ: 5 ng L^{-1}	Influent wastewater (STP)	SPE	10 – 95 ng L^{-1} mean: 95 ± 29 ng L^{-1}	—	—	GC/LC-MS GC/LC-MS/MS	[84]
		Effluent wastewater (STP)		1 – 8 ng L^{-1} mean: 3 ± 2 ng L^{-1}				
		Upstream river		0 – 3 ng L^{-1} mean: 1 ± 2 ng L^{-1}				
		Downstream river		1 – 4 ng L^{-1} mean: 2 ± 1 ng L^{-1}				

	Competitive ELISA	LOQ: 0.05 ± 0.01 ng L^{-1} (EE2/MeEE2)	Fresh surface water (river samples)	SPE and enrichment	4.1 – 8.3 ng L^{-1} 15 – 29 ng L^{-1}	Salinity Humic Acids (HA) Organic solvents: ethanol and methanol CR	CR: Ethinylestradiol-3- methyl ether (mestranol, MeEE2) (118%)	GC-MS (R^2: 0.934)	[95]
Estriol (E3)	ELISA kit	LOD: 1 ng L^{-1} LOQ: 5 ng L^{-1}	Influent wastewater (STP)	SPE	3 – 9 ng L^{-1} mean: 9 ± 2 ng L^{-1}	—	—	GC/LC-MS GC/LC-MS/MS	[84]
			Effluent wastewater (STP)		< 1 ng L^{-1} mean: < 1 ng L^{-1}				
			Upstream river		< 1 ng L^{-1} mean: < 1 ng L^{-1}				
			Downstream river		< 1 – 2 ng L^{-1}				
Progesterone	ELISA kit	LOD: 3 ng L^{-1} LOQ: 5 ng L^{-1}	Influent wastewater (STP)	SPE	163 – 904 ng L^{-1} mean: 408 ± 220 ng L^{-1}	—	—	GC/LC-MS GC/LC-MS/MS	[84]
			Effluent wastewater (STP)		0 – 25 ng L^{-1} mean: 9 ± 8 ng L^{-1}				
			Upstream river		0 – 12 ng L^{-1} mean: 7 ± 6 ng L^{-1}				
			Downstream river		0 – 60 ng L^{-1} mean: 13 ± 19 ng L^{-1}				
Testosterone	ELISA kit	LOD: 2 ng L^{-1} LOQ: 5 ng L^{-1}	Influent wastewater (STP)	SPE	119 – 635 ng L^{-1} mean: 343 ± 226 ng L^{-1}	—	—	GC/LC-MS GC/LC-MS/MS	[84]
			Effluent wastewater (STP)		0 – 26 ng L^{-1} mean: 11 ± 7 ng L^{-1}				
			Upstream river		5 – 16 ng L^{-1}				

		mean: 10 ± 6 ng L^{-1}
	Downstream river	3 – 19 ng L^{-1}
		mean: 10 ± 6 ng L^{-1}

BSA - bovine serum albumin; CR – cross reactivity; HA – humic acids; QR – quantification range (allows a maximum relative error of 10%); WR – working range (allows a maximum relative error of 30%) ; LOD – Detection limit; IC_{50} – half maximum inhibitory concentration; SPE – solid-phase extraction; DLLME – Dispersive liquid-liquid microextraction; C_{VT} – concentration measured by the validation technique; C_{ELISA} – concentration measured by ELISA method; N.D. – non detectable; POCIS – polar organic compound integrated sampler

Table 4. Direct competitive ELISA for the detection of pharmaceuticals in biological samples.

Pharmaceutical	Quantification limits and linear range	Species	Sample pre-treatment	Levels detected (fresh weight) at the end of the exposure assay	References
Carbamazepine (CBZ)	QR: 0.03–10 µg L^{-1} (method based on Calisto et al. [66])	*Venerupis decussata* (clam)	Pulverized clam frozen tissues, extracted with deionized water	< LOQ (E.C.: 0.00 µg L^{-1}) < LOQ (E.C.: 0.03 µg L^{-1}) 0.00008 µg g^{-1} (E.C.: 0.30 µg L^{-1}) 0.0014 µg g^{-1} (E.C.: 3.00 µg L^{-1}) 0.010 µg g^{-1} (E.C.: 9.00 µg L^{-1})	[118]
		Venerupis philippinarum (clam)		< LOQ (E.C.: 0.00 µg L^{-1}) < LOQ (E.C.: 0.03 µg L^{-1}) 0.000034 µg g^{-1} (E.C.: 0.30 µg L^{-1}) 0.0006 µg g^{-1} (E.C.: 3.00 µg L^{-1}) 0.0109 µg g^{-1} (E.C.: 9.00 µg L^{-1})	
	LLOQ: 0.024 µg L^{-1} (method described Almeida et al. [118])	*Ruditapes philippinarum* (clam)	Mechanical pulverization of soft tissues (of frozen organisms) using liquid nitrogen sonication and centrifugation Pulverized tissues extracted with deionized water	< LOQ (E.C.: 0.00 µg L^{-1}) 0.03 ng g^{-1} (E.C.: 0.03 µg L^{-1}) 0.23 ng g^{-1} (E.C.: 0.30 µg L^{-1}) 3.6 ng g^{-1} (E.C.: 3.00 µg L^{-1}) 5.9 ng g^{-1} (E.C.: 9.00 µg L^{-1})	[115]
	LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Scrobicularia plana* (clam)	Pulverized clam frozen tissues, extracted with deionized water	CBZ: Between 2.5 – 3.0 ng g^{-1} * (E.C.: 3.00 µg L^{-1}) CBZ+pH 7.1: Between 1.5 – 2.0 ng g^{-1} * (E.C.: 3.00 µg L^{-1})	[119]

LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Scrobicularia plana* (clam)	Pulverized clam frozen tissues, extracted with deionized water	< 0.5 ng g^{-1} * (E.C.: 0.30 µg L^{-1}) ~ 3 ng g^{-1} * (E.C.: 3.00 µg L^{-1}) ~ 6 ng g^{-1} * (E.C.: 6.00 µg L^{-1}) ~ 8 ng g^{-1} * (E.C.: 9.00 µg L^{-1})	[116]
	Diopatra neapolitana (polychaete)		~ 2 ng g^{-1} * (E.C.: 0.30 µg L^{-1}) ~ 5 ng g^{-1} * (E.C.: 3.00 µg L^{-1}) ~ 8 ng g^{-1} * (E.C.: 6.00 µg L^{-1}) ~ 12 ng g^{-1} * (E.C.: 9.00 µg L^{-1})	
LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Scrobicularia plana* (clam)	Pulverization of whole soft tissues and extraction with deionized water	For non-irradiated CBZ: < 1 ng g^{-1} * (E.C.: 0.3 µg L^{-1}) ~ 5 ng g^{-1} * (E.C.: 3.0 µg L^{-1}) ~ 7 ng g^{-1} * (E.C.: 6.0 µg L^{-1}) ~ 13 ng g^{-1} * (E.C.: 9.0 µg L^{-1}) For irradiated CBZ: < 1 ng g^{-1} * (E.C.: 0.3 µg L^{-1}) ~ 4 ng g^{-1} * (E.C.: 3.0 µg L^{-1}) ~ 7 - 8 ng g^{-1} * (E.C.: 6.0 µg L^{-1}) ~ 12 ng g^{-1} * (E.C.: 9.0 µg L^{-1})	[113]
LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Scrobicularia plana* (clam)	Mechanical pulverization of the soft tissues (of frozen organisms); sonication and centrifugation; extraction with deionized water	< 0.5 ng g^{-1} * (E.C.: 0. 0 µg L^{-1}) ~ 4 - 6 ng g^{-1} * (E.C.: 4 µg L^{-1}) ~ 7 - 10 ng g^{-1} * (E.C.: 8 µg L^{-1})	[120]
QR: 0.03 -10 µg L^{-1} (method based on Calisto et al. [66])	*Mytilus galloprovincialis* (mussel)	Mechanical pulverization of the frozen soft tissues; sonication; centrifugation; extraction with deionized water	Chronic exposure (28 days): 0.6 ng g^{-1} (E.C.: 0.3 µg L^{-1}) 4.9 ng g^{-1} (E.C.: 3.0 µg L^{-1}) 10.9 ng g^{-1} * (E.C.: 6.0 µg L^{-1}) 13.0 ng g^{-1} * (E.C.: 12.0 µg L^{-1})	[54]

	LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Ruditapes philippinarum* (clam)	Extraction with deionized water; sonication; centrifugation	2.0 ng g^{-1} (E.C.: 1.0 µg L^{-1})	[112]
	LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Ruditapes philippinarum* (clam)	Sonication; centrifugation; pooled gills extracted with deionized water	1.8 ng g^{-1} (E.C.: 1.0 µg L^{-1})	[111]
Cetirizine (CET)	LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Ruditapes philippinarum* (clam)	Pulverization of the soft tissues; extraction with deionized water	< 0.5 ng g^{-1} * (E.C.: 0.3 µg L^{-1}) 0.3 ng g^{-1} * (E.C.: 0.3 µg L^{-1}) ~ 2 ng g^{-1} * (E.C.: 3.0 µg L^{-1}) ~ 5 ng g^{-1} * (E.C.: 6.0 µg L^{-1}) ~ 9 ng g^{-1} * (E.C.: 12.0 µg L^{-1})	[121]
	LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Ruditapes philippinarum* (clam)	Extractions performed in deionized water; sonication; centrifugation	2.9 ng g^{-1} (E.C.: 0.6 µg L^{-1})	[112]
	LLOQ: 0.024 µg L^{-1} (method described in Almeida et al. [118])	*Ruditapes philippinarum* (clam)	Extraction of the pooled gills with deionized water; sonication; centrifugation	2.0 ng g^{-1} (E.C.: 0.6 µg L^{-1})	[111]

E.C. – exposure concentration (theoretical value); LLOQ – Lower limit of quantification; LOQ – Limit of quantification; QR – Quantification range
* values taken from graph (approximated value)