Ecotoxicology and Environmental Safety 144 (2017) 585–592

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

A survey of 17α-ethinylestradiol and mestranol residues in Hawkesbury River, Australia, using a highly specific enzyme-linked immunosorbent assay (ELISA) demonstrates the levels of potential biological significance

Chatchaporn Uraipong[a], Robin D. Allan[b], Chunhua Li[c], Ivan R. Kennedy[c], Victor Wong[a], Nanju Alice Lee[a,*]

[a] School of Chemical Engineering, Food Science and Technology, the University of New South Wales, Sydney, NSW 2052, Australia
[b] Department of Pharmacology, the University of Sydney, Sydney, NSW 2006, Australia
[c] Faculty of Agriculture, Food and Natural Resources, the University of Sydney, Sydney, NSW 2006, Australia

ARTICLE INFO

Keywords:
Hapten synthesis
ELISA
Endocrine disrupting hormones
17α-ethinylestradiol
Mestranol
Water monitoring

ABSTRACT

This study reports on the potential status of 17α-ethinylestradiol (EE2) and mestranol (MeEE2) residues in aquatic environments in New South Wales (NSW), Australia, based on the analysis by a specific ELISA we developed. Polyclonal antibodies were raised against the EE2 hapten with a linker attached at the C3-position to direct the antibody binding towards the ring D of EE2/MeEE2. Using this approach, an ELISA highly specific to EE2 and MeEE2 was successfully developed, showing less than 3.1% cross-reactivity (% CR) with other major steroidal sex hormones and their derivatives. The assay performed with the limit of detection (LOD) of 0.04 ± 0.01 μg/L for both EE2 and MeEE2, and the limit of quantitation (LOQ) of 0.05 ± 0.01 ng/L when it was coupled with the SM2-Biobeads solid phase extraction. Prior to conducting the survey study, it was validated against the gas chromatography-mass spectrophotometry (GC-MS) method, which showed high correlation with R^2 of 0.934. Fresh surface water samples collected at different sites along Hawkesbury River in New South Wales (NSW) were analyzed for the EE2/ MeEE2 residues using the developed ELISA. The EE2/MeEE2 levels were found to range between 4.1 and 8.3 ng/L in Emigrant Creek, NSW, where the primary activity was macadamia plantation, and higher levels between 15 and 29 ng/L in South Creek, NSW, Greater Western Sydney at sites upstream and downstream of the municipal sewage treatment plants.

1. Introduction

Water is a precious resource, particularly in drought-prone countries with unpredictable weather patterns such as Australia. The strategy of introducing treated recycled water into the portable water supply system remains attractive for general households and agricultural purposes. However, the use of recycled water is not without issues; of great concern is the environmental release of residual chemicals that may induce adverse effects on natural ecosystems and, subsequently, the entire food chain. In recent years, a lot of research interests has been directed toward studying chemicals that possess the ability to mimic and/or disrupt the endocrine system by altering the natural balance of hormones in the human body. These compounds have been termed endocrine disrupting compounds (EDCs) as their biological and ecological activities interrupt the proper functioning of the endocrine system of animals (Colborn et al., 1993; Oberdorster and Cheek, 2001).

The natural estrogens include estrone (E1), estradiol (E2) and estriol (E3) have known endocrine disrupting properties, and are introduced into the environment such as rivers, lakes, and sea water through wastewater treatment effluents, and their concentration in the environment generally ranked at 0.83 – 150,000 ng/L (Gutendorf and Westendorf, 2001; Adeel et al., 2017). These steroidal hormones have been detected at higher than biologically significant levels in municipal and industry effluents in Europe, North America and Asia (Ternes et al., 1999; Oh et al., 2000; Carvalho et al., 2016). In Australia, only a few studies have been conducted on EDCs, mostly in Queensland, Victoria and South Australia (Game et al., 2006; Williams et al., 2007; Gadd et al., 2010), and very limited studies in New South Wales (Scott et al., 2014). This demonstrated a need for more comprehensive studies and for assessing non-point sources, such as industrial and agricultural inputs.

The synthetic hormones, EE2 and mestranol (MeEE2), are the most common synthetic chemicals found in oral contraceptives. MeEE2, an

* Corresponding author.
E-mail address: alice.lee@unsw.edu.au (N.A. Lee).

http://dx.doi.org/10.1016/j.ecoenv.2017.06.077
Received 3 March 2017; Received in revised form 12 June 2017; Accepted 30 June 2017

inactive prodrug of EE2, is converted to EE2 by demethylation in mammalian liver. EE2 has been detected at trace levels in the low ng/L range in sewage effluents and surface water (Vieira Dias et al., 2015; Avar et al., 2016). Recent observations of EE2 exposure (4 - 10 ng/L) affected the development of aquatic organism, including the gonadal differentiation in zebrafish (Luzio et al., 2016a), increasing brain aromatase activity in copper redhorse (Maltais and Roy, 2014), and the inhibition of gonadal and spermatogenic development in Sydney rock oysters (Andrew et al., 2008).

MeEE2 have been reported on both wastewater and surface water at 2.7–700 ng/L (Baronti et al., 2000; Carvalho et al., 2016). Recently, feminization in the fin morphometry of male Thai rice fish exposed to 10 mg/L MeEE2 has been observed (Ngamniyom et al., 2012). The synthetic estrogenic steroids such as EE2 and mestranol have lower water solubility, suggesting longer persistence in the environment. They are hydrophobic organic compounds, and more susceptible to biodegradation in water, but they may become resistance to biodegradation by partitioning in sediments and soil (Ternes et al., 1999; Adeel et al., 2017).

Regulations of endocrine disrupting estrogens have yet been globally harmonized, although many regulatory bodies recognize the issue and suggest further monitoring. The European Union (EU) does not include EE2 in water legislation for human consumption but had included in the watch list on Directive 2013/39/EU for environmental quality standards applicable to surface water (Cunha et al., 2016). On the other hand, EE2 is regulated in the U.S drinking water standard, but it does not include on the list of quality control standards for environmental water (Cunha et al., 2016). No official regulations of synthetic estrogens have been implemented in Australia.

Significant research efforts have been devoted to addressing the analytical challenges of quantifying EDCs at ng/L levels. Complementary to the sophisticated hyphenated instrumentations such as gas chromatography-mass spectrometry (LOQ = 0.08 - 80.0 ng/L) (GC-MS) (Zacs et al., 2016), gas chromatography-tandem mass spectrometry (GC-MS/MS) (LOQ = 0.2 ng/L) (Avar et al., 2016), liquid chromatography-mass spectrometry (LC-MS) (LOQ = 0.02 - 0.22 μg/L) (Céspedes et al., 2004) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (LOQ = 3 - 15 ng/L) (Naldi et al., 2016), immunochemical assays such as ELISA offers alternative analytical advantages such as high-throughput capacity, being capable of analysing a greater number of samples at a lower cost which is particularly beneficial for large-scale screening purposes (Lee and Kennedy, 2007). In addition, ELISAs can be used for the identification and quantification of specific analytes within a crude mixture owing to highly specific nature of the antibody to its antigen. The technique of ELISA had been employed in determining EE2 in environmental and industrial waste samples. For example, the commercial ELISA test kits (typically developed for biomedical applications) have been applied for environmental analysis either with or without proper validation, which have shown to display the lowest quantification limit of 5 ng/L (Manickum and John, 2015), 50 ng/L (Hirobe et al., 2006) and 0.12 ng/L (Hintemann et al., 2006) after an extensive enrichment or concentration step is performed.

One of the immunochemical criteria particularly important to the detection of steroidal sex hormones is specificity, which may require different analytical criteria for environmental analysis. This paper is divided into two aspects: 1) the development and characterization of a sensitive enzyme-linked immunosorbent assay for specific detection of EE2 and MeEE2 using the novel hapten design and synthesis, and 2) the validation and application of the developed ELISA in an environmental survey of EE2/MeEE2 residues in water supply such as drinking water and agricultural activities for New South Wales, Australia. The paper also demonstrates the value of the developed assay for time and cost effective monitoring of EDC residues in our precious water resources.

2. Materials and methods

2.1. Materials and instruments

17α-Ethinylestradiol (EE2), 17β-estradiol (E2), estriol (E3), estrone (E1), estradiol dipropionate, progesterone, medroxyprogesterone, ethinylestradiol-3-methyl ether, 17α-ethinylestradiol-3-cyclopentyl ether, *N*-hydroxysuccinimide (NHS), humic acid, dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), Estradiol-one-6-*O*-carboxymethyloxime (E2-CMO), and estradiol-3-hemisuccinate (E2-HS) were purchased from Sigma-Aldrich (St. Louis, USA). Dimethylformamide (DMF) and tetrahydrofuran (THF) were obtained from Ajax Finechem (Sydney, Australia). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), horseradish peroxidase (HRP), goat-anti-rabbit IgG, Tween 20, thiomersal, TiterMax Gold and incomplete Freund's adjuvant were purchased from Sigma (St. Louis, USA). For the preparation of buffers, chemicals were sourced from either BDH Chemicals (Melbourne, Australia) or Ajax Finechem (Sydney, Australia). Maxisorp polystyrene 96-well plates were obtained from Nunc (Roskilde, Denmark). HiTrap™ protein-A HP column was purchased from GE Healthcare (Bellefonte, USA). The ELISA plate reader (SpectroMax M2) was obtained from Molecular Devices (Sunnyvale, USA).

2.2. Hapten synthesis

The use of saturated linkers of varying carbon lengths attached to the hydroxyl group of EE2/E2, such as an acetate linker (for 3 carbon chain) and a butyrate linker (4 carbon chain), were employed in the hapten synthesis (Fig. 1). The reaction pathways for the synthesis of EE2 and E2 haptens are presented in Supplementary Fig. S1 and S2.

2.3. Preparation of immunogens and enzyme conjugates

The immunogen was prepared by conjugating a carrier protein (KLH) and a 17α-ethinylestradiol-3-acetate (denoted as EE2-ACT-KLH). The competing hapten-enzyme conjugates were prepared by conjugating HRP with a series of haptenic analogs presented in Fig. 1. All conjugations of haptens and protein/enzyme were performed via the NHS/DCC active ester reaction as follows: Each active ester dissolved in dry DMF was added dropwise to a protein dissolved in a pre-cooled buffer solution (50 mM K_2HPO_4, pH 9.1). The reaction solution was gently mixed and left to stand at 4 °C overnight. The conjugate solution was then dialyzed against phosphate buffered saline (PBS, pH 7.2) and the resultant solution was stored at 4 °C until use.

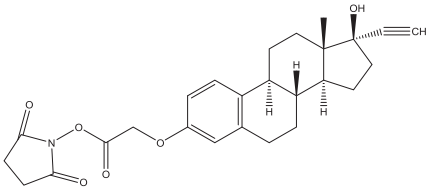
E2-CMO and E2-HS were also conjugated to an enzyme separately via the NHS/DCC active ester method. The estrone-3-hemisuccinate (E1-HS) active ester was prepared according to Li et al. (2004).

2.4. Antibody production

New Zealand white rabbits were immunized with the immunogen, EE2-ACT-KLH, by subcutaneous injections. The rabbits were given the immunogen containing EE2-ACT-KLH in 0.9% NaCl (saline) (0.5 mL) emulsified with either TiterMax adjuvant or Freund's incomplete adjuvant (0.5 mL). Subsequent booster injections were given at monthly intervals. Blood was collected from the marginal ear vein 8–10 days after each injection, and the serum (supernatant) was collected after centrifuging at ×1500*g* for 15 min.

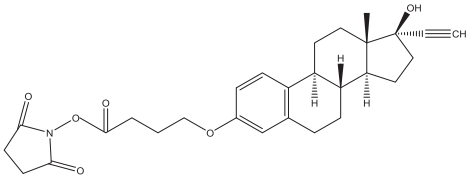
2.5. Preparation of standard solution

Standard stock solutions (either in 100 mg/L or in 10 mg/L) of EE2 and its analogs (E2, E1, E3, estradiol dipropionate, progesterone,



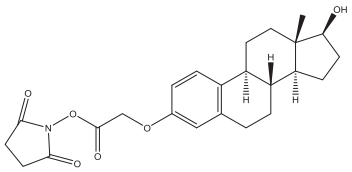
17α-ethinylestradiol-acetate

(EE2-ACT-HRP)

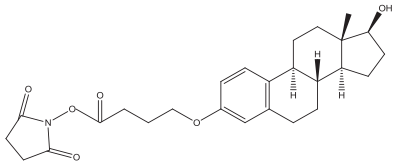


17α-ethinylestradiol-butyrate

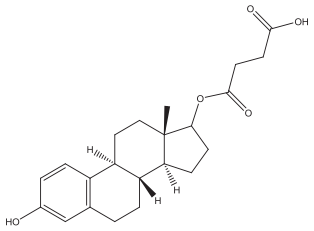
(EE2-BUT-HRP)



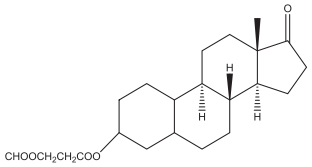
17β-estradiol-acetate (E2-ACT-HRP)



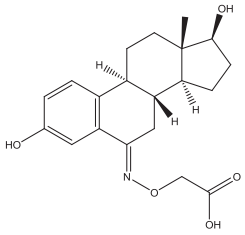
17β-estradiol-butyrate (E2-BUT-HRP)



17β-estradiol-hemisuccinate (E2-HS-HRP)



Estrone-3-hemisucciante (E1-HS-HRP)



17β-estradiol-one6-*O*-carboxymethyl-oxime

(E2-CMO-HRP)

Fig. 1. The chemical structures of haptenic molecules used in the assay development.

medroxyprogesterone, ethinylestradiol-3-methyl ether and 17α-ethinylestradiol-3-cyclopentyl ether) were prepared by dissolving the respective amount of each chemical in ethanol (EtOH). The individual stock solutions were stored at 4 °C in amber glass vials. A working standard was freshly prepared by diluting the stock solution to give a 100 μg/L standard solution (in 10% EtOH). This solution was then serially diluted with 10% EtOH to obtain the concentrations between 0.01–33.3 μg/L.

2.6. Competitive ELISA

Microwell plates were coated with the purified anti-EE2 IgG at 1 μg per well in 50 mM carbonate buffer, pH 9.6. Microplates were then washed three times with a washing solution (0.05% Tween 20), then dried on an absorbent paper. The unoccupied sites on the microwells were blocked with 1% fish gelatine in PBS (1% FG/PBS). Then, the plate was washed and dried as previously described. For the control, the wells were loaded with 100 μL of 10% EtOH and 100 μL of enzyme conjugate. For the blank, the respective wells were loaded with 100 μL of 10% EtOH and 1% BSA/PBS (an enzyme conjugate diluent). To construct a standard curve, standard solutions plus an enzyme conjugate solution were applied to the allocated wells and the microplates were incubated for 1 h. Similarly, a sample plus an enzyme conjugate solution were applied to the respective wells. Following washing, a

substrate solution (1.25 nM 3,3′,5,5′-tetramethylbenzidine/1.6 mM hydrogen peroxide, pH 5.0) was added to each well. The color reaction was developed for 30 min and then stopped by adding 50 µL of 1.25 M H_2SO_4. Absorbance was measured using SpectroMax M2 microplate reader with a dual wavelength mode at 450 and 650 nm.

2.7. Cross-reactivity

The relative sensitivity of immunoassay towards the compounds with structural similarity was determined by comparing calibration curve of each test hormone with EE2. The cross-reactivity was calculated as the ratio of concentrations at the IC_{50} values of the respective calibration curves and expressed in the percentage relative to that of the EE2 curve. The percent cross-reactivity (% CR) was calculated using the following equation:

$$\%CR = \frac{IC_{50(EE2)}}{IC_{50(testcompound)}} \times 100 \quad (1)$$

2.8. Effects of matrix interference

Assay stability was evaluated against environmental factors such as ionic strengths of NaCl (to simulate the effects of sea water), the presence of humic acid and pH that may affect assay performance. For the comparison, EE2 standard solutions were prepared in the different ionic strengths of NaCl between 0 and 0.1 M in 10% EtOH. Humic acid was tested because of its properties as absorbents and ion exchangers in aquatic environments. The effect of humic acid was investigated at the concentrations ranging from 0.1 to 1000 µg/L. The effect of pH was evaluated at various pH values (3, 5, 8, 9, 10 and 11) and compared with the control pH at 7.2.

2.9. The validation of ELISA with the validated GC/MS

The ELISA was validated by the GC/MS method that was previously validated in our laboratory, using the spiked samples (Li et al., 2007). The assays were independently conducted in the two different laboratories using different EE2 standards. Briefly, 8 L of MilliQ purified water sample spiked in duplicates with EE2 between the concentrations of 10 and 50 ng/L, were extracted and concentrated to a final volume of 10 mL dichloromethane (DCM) (Li et al., 2007). The aliquots (1 mL) were subsequently reduced to dryness under N_2 at 60°C prior to dissolution in 1 mL of 10% MeOH for the immunoassay. For the GC/MS analysis, Agilent 6890 gas chromatography coupled to a 5973 mass spectrometry (USA) was used. Samples were separated on a 0.25 mm × 30 m × 0.25 µm DB-1701 capillary column (Agilent, USA). The column temperatures were programmed as follows: the initial oven temperature was 80 °C, increased to 280 °C with a ramp rate of 20 °C per min. The total running time was 30 min. Helium was used as a carrier gas with the pressure 11.06 psi (on), purge flow 0.5 mL/min, purge time 2.00 min and total flow 8.7 mL/min. The injector temperature was maintained at 230 °C and the injector volume was 4 µL in splitless inlet mode. Gas flow was constant with an initial flow of 1.1 mL/min under a nominal initial pressure 11.07 psi. Chemstation Software was used for the calculation of data.

2.10. Survey of EE2/MeEE2 residues in the environmental water samples collected from South Creek and Emigrant Creek, New South Wales

Water samples were collected from South Creek, Sydney and Emigrant Creek, Northern New South Wales, Australia (Li et al., 2007). A total of eight 8 L samples were collected upstream of the discharge from the urban wastewater treatment plant (WWTP), at the discharge point and downstream of the discharge point. The water samples were subjected to the extraction and enrichment procedure described in Section 2.9. The concentrated extracts were analyzed in duplicates by the ELISA with EE2 as a standard.

3. Results and discussions

3.1. Hapten synthesis and hapten selection

The goal of this study was to develop the highly specific polyclonal antibody for the detection of EE2 (and MeEE2) residues in environmental samples. The specificity of the antibodies produced towards the targeted steroids is significantly related to the orientation of the hapten presented to the immune system upon immunization (i.e., a moiety of the hapten molecule that is attached to the carrier protein). All estrogenic hormones share the same backbone structure consisting of 4 rings, differing only in their substituent groups on C17 and C18. Therefore, our rationale for designing a series of haptens with the linker attachment on C3 is to induce an immunogenic response towards the most variable moiety (i.e., ring D) on EE2 and MeEE2. This mode of conjugation would preserve the acetylene substituent group on the ring D, potentially leading to the generation of polyclonal antibodies able to differentiate EE2/MeEE2 from other steroids such as 17β-estradiol, estrone, and estriol. Hapten in this study was designed differently from the previously reported EE2 ELISAs. For example, Hintemann et al. (2006) produced the EE2 polyclonal antibodies using a BSA conjugate of the EE2-6-carboxymethyloxime, and Schneider et al. (2004) raised long-chain biotinylated EE2 specific antibodies using a BSA conjugate of 1,3,5(10)estratrien-17-ethynyl-3,17-diol-6-one-6-carboxymethyloxime (EE2-6-CMO). These haptens represent the linker attachment at the C6 positions.

We chose the EE2 with an acetate linker conjugated to KLH as an immunogen for raising specific polyclonal antibodies. The short saturated 3-carbon chain linker ensured that the flexibility of the hapten molecule after the conjugation to the carrier molecule was maintained. The flexibility of the saturated linker allowed for greater interaction with the immune system upon immunization, and less likelihood of triggering an immunogenic response against the linker. Equally important is that the aqueous solubility of the hapten as well as the possibility of a hapten to fold back towards and into the carrier protein, thus reducing the chances for immune system recognition (Lee and Kennedy, 2007), although moderately hydrophobic haptens tend to elicit better immune responses in our experience.

3.2. Screening for the best the antibody-enzyme conjugate combinations

A series of haptens used for the preparation of enzyme/protein conjugates included our newly synthesized haptens, 17α-ethinylestradiol-acetate (EE2-ACT), 17α-ethinylestradiol-butyrate (EE2-BUT), 17β-estradiol-acetate (E2-ACT), 17β-estradiol-butyrate (E2-BUT), and a previously synthesized hapten, estrone-3-hemisuccinate (E1-HS, Li et al., 2004, Fig. 1). The two commercially available haptens used in this study were 17β-Estradiol-one6-O-carboxymethyl-oxime (E2-CMO) and 17β-estradiol-hemisuccinate (E2-HS) (Fig. 1). These haptens were conjugated to HRP to be used as competing species in a competitive ELISA. EE2-ACT, EE2-BUT, E2-ACT, and E2-BUT were conjugated to HRP. This gave rise to a total of 7 enzyme conjugates, denoted as EE2-ACT–HRP, EE2-BUT–HRP, E2-ACT–HRP, E2-BUT–HRP, E2-HS-HRP, E2-OX-HRP and E1-HS-HRP to be evaluated in this study.

The antibodies against the EE2-ACT-KLH immunogen were denoted as Ab∝EE2-ACT1, Ab∝EE2-ACT2 (1 and 2 indicate host animal 1 and host animal 2). Optimum conditions for each enzyme-conjugate were assessed by conducting the checkerboard titration against immobilized antibodies and selecting the optimum concentrations of the enzyme conjugates. Ab∝EE2-ACT generated 4-fold higher titers in the homologous assay than in the heterologous assay, and it did not show adequate absorbance when titrated against E2-CMO-HRP (data not shown). Hence, no further evaluation was conducted for Ab∝EE2-ACT/ E2-CMO-HRP. Future characterization of the heterologous assay was

Table 1
The IC_{50} values and maximum absorbance values of the optimized calibration curves of the ELISA using Ab∝EE2-ACT1#4 with 7 different hapten-enzyme conjugates.

Enzyme conjugates	Ab∝EE2-ACT1#4	
	IC_{50} (μg/L)	Maximum Absorbance (Ab unit)
17α-ethinylestradiol-acetate (EE2-ACT-HRP)	7.4 ± 2.1	1.2 ± 0.1
17α-ethinylestradiol-butyrate (EE2-BUT-HRP)	3.9 ± 1.0	1.2 ± 1.2
17β-estradiol-acetate (E2-ACT-HRP)	0.3 ± 0.1	0.9 ± 0.2
17β-estradiol-butyrate (E2-BUT-HRP)	0.4 ± 0.1	0.6 ± 0.2
17β-estradiol-hemisuccinate (E2-HS-HRP)	4.0 ± 1.2	0.6 ± 0.2
Estrone-3-hemisucciante (E1-HS-HRP)	2.2 ± 0.1	0.7 ± 0.5
17β-estradiol-one6-*O*-carboxymethyl-oxime (E2-CMO-HRP)	NA[a]	0.1 ± 0.02

[a] Low color development on the assay and subsequently no study on the assay sensitivity.

carried out to evaluate assay sensitivity.

3.3. Assay sensitivity

In our study, Ab∝EE2-ACT1 was selected for further characterization and optimization with the competing haptens in a competitive assay. Additionally, the sensitivity of different bleeds of each antibody was monitored to evaluate the antibody maturation. The fourth and later bleeds proved to yield better titer and sensitivity (Table 1).

As shown in Table 1, the absolute homologous systems (i.e., both hapten and linkage are the same), employing Ab∝EE2-ACT1#4 with EE2-ACT-HRP, exhibited the IC_{50} values higher than the assays employing the hapten-homology with the linker-heterology approach (i.e., Ab∝EE2-ACT1#4/EE2-BUT-HRP). As expected, the heterologous assays were able to further improve the sensitivity, but not all heterologous assays showed an improved sensitivity. The hapten heterologous systems of Ab∝EE2-ACT1#4/ E2-ACT-HRP, or E2-BUT-HRP, demonstrated significantly increased sensitivity with the IC_{50} values ranging from 0.3 to 0.9 μg/L. In contrast, the assay based on Ab∝EE2-ACT1#4/ E2-HS-HRP presented low sensitivity (i.e., higher IC_{50} values). As well, the relatively high concentrations of enzyme conjugates E2-HS-HRP, E2-CMO-HRP and E1-HS-HRP needed in the absolute heterologous assays made them impractical for routine analyses, though it did not increase the background color, and further characterization was not conducted. The best sensitivity was provided by the Ab∝EE2-ACT1#4/ E2-ACT-HRP pair, with an IC_{50} value of 0.3 ± 0.1 μg/L EE2 and the detection range of 0.04 ± 0.02 – 1.7 ± 0.6 μg/L EE2.

Our assay displayed a similar sensitivity as those assays with the colorimetric measurement reported by Hirobe et al. (2006) and Schneider et al. (2004), which displayed the detection range of 0.05–3.0 μg/L and 0.02 – 1.2 μg/L, respectively. Of the mentioned assays, only the ELISA reported by Schneider et al. (2005) using the chemiluminescence measurement yielded a higher sensitivity assay with the analytical working range of 0.8 – 100 ng/L.

3.4. Assay specificity

Overall, the cross-reactivity of the tested major steroidal sex hormones and their derivatives did not exceed 3.1% cross-reactivity (Table 2). For estriol, estrone, estradiol dipropionate, progesterone, 17α-estradiol and medroxyprogesterone, the % cross–reactivity was less than 0.5%, showing higher specificity than the previously reported assays. The assay showed slightly higher cross-reactivity of E2 (3.1%) than the previous studies, but it was still lower than our criterion of 5% cross-reactivity (Schneider et al., 2004, 2005). The % cross-reactivity of ethinylestradiol-3-methyl ether (mestranol, MeEE2) was 118% as expected due to the position of the linker attachment.

Table 2
The IC_{50} and % cross-reactivity (% CR) for the selected steroidal compounds.

Compounds	Cross-Reactivity	
	IC_{50} (mol/L)	% CR
17α-ethinylestradiol	1.3×10^{-9}	100
Ethinylestradiol-3- methyl ether (mestranol)	1.1×10^{-9}	118
17β-estradiol	4.1×10^{-8}	3.1
Estriol	3.5×10^{-7}	0.4
Estrone	3.7×10^{-7}	0.4
Estradiol dipropionate	2.6×10^{-7}	0.5
Progesterone	3.2×10^{-7}	0.4
17α-estradiol	3.7×10^{-7}	0.4
Medroxyprogesterone	2.9×10^{-7}	0.5

Our hapten design with the linker attached at the C3-position indeed directed the antibody binding towards the ring D of EE2, resulting in a highly specific ELISA to EE2 with negligible cross-reaction with other steroidal sex hormones with various substituent groups at the C17-position. The assay detected equally well MeEE2 which exhibited a similar structure with OCH_3 on the C3-position. Thus, the Ab∝EE2-ACT based assay is capable of quantifying both EE2 and MeEE2 residues in environmental samples.

Effects of organic solvents, pH, ionic strength of NaCl (salinity) and humic acid. Maintaining total dissolution of the hydrophobic EE2 in the aqueous calibration solutions is critical for the immunoassay consistency. Methanol (MeOH) and EtOH are generally preferred for extraction and concentration of EE2 from various matrices. For this reason, the effect of organic solvents on the ELISA was evaluated by comparing the EE2 calibration curves prepared in MeOH, EtOH, acetone, and acetonitrile at different concentrations. The assay could only tolerate up to 10% (v/v) MeOH and EtOH. However, concentrations greater than 10% MeOH/ EtOH either inhibited the enzyme activity of the hapten-enzyme conjugates or interfered with the antibody-antigen interaction or both of these effects, judged by the color reduction and/or the loss of sensitivity (Supplementary Fig. S3). Based on the above findings, 10% EtOH was chosen for all subsequent assays.

Proteins, such as antibodies and enzymes may be susceptible to pH fluctuation. Assay performance was evaluated at pH ranging from 3 to 11. Both the inhibitions of enzyme activity and the antibody-antigen binding in all the assays were observed with pH lower and higher than 7.2. The solvent at pH values of 3, 5, 9, 10 and 11 significantly increased the IC_{50} values from 0.3 ± 0.01 μg/L (at the control pH 7.2) to 0.9 ± 0.4 μg/L, 0.4 ± 0.04 μg/L, 0.9 ± 0.1 μg/L, 0.8 ± 0.1 μg/L, and 3.3 ± 0.4 μg/L, respectively. No significant change in the IC_{50} value was observed at pH 8 (0.3 ± 0.02 μg/L). The study found that our antibodies were sensitive to pH, only performing best around pH 7–8, and the pH of samples must be adjusted prior to analysis in order to minimize analytical variations.

The effect of ionic strength of NaCl as the test of salinity on the assay performance was conducted. The NaCl concentrations lower than 0.01 M had negligible effects on the assay sensitivity. The IC_{50} values increased (i.e., decrease in sensitivity) with increasing NaCl concentrations approaching 0.1 M. Using 10% EtOH with no NaCl, 0.001 M NaCl, 0.01 M NaCl, and 0.1 M NaCl as a matrix, the IC_{50} values increased to 0.2 ± 0.1 μg/L, 0.3 ± 0.1 μg/L, 0.5 ± 0.1 μg/L and 3.6 ± 0.1 μg/L, respectively. The maximum absorbance value, however, was not affected at the tested ionic strengths and it diminished only slightly at 0.1 M NaCl. From the observation, it was recommended to reduce the salinity to less than 0.01 M for the optimum assay performance.

A heterogeneous mixture of organic acids such as humic acid is often present in a range from low μg/L to several hundred mg/L in soils and water sediments (Senesi and Miano, 1994; Boenigk et al., 2005).

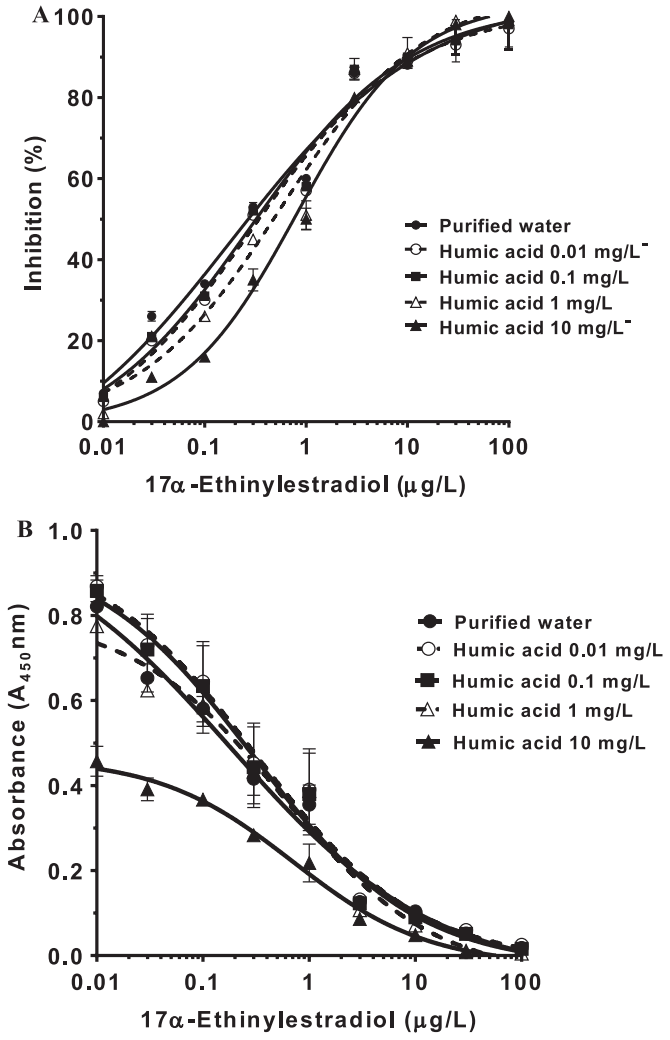


Fig. 2. Effects of humic acids on the ELISA based on Ab∝EE2-ACT1#4 (antibody) and E2-ACT12-HRP (HRP conjugate). The standard curves are represented in (A) % inhibition vs concentration and (B) absorbance units vs concentration; • = no humic acid in 10% EtOH; ▲ = humic acid at 10 mg/L in 10% EtOH; △ = humic acid at 1 mg/L in 10% EtOH; ■ = humic acid at 0.1 mg/L in 10% EtOH; ○ = humic acid at 0.01 mg/L in 10% EtOH. Data are means of duplicate analysis ± SD (n = 2).

They are regarded as natural absorbents, ion exchangers, biochemical regulators, and reservoirs. Consequently, the presence of naturally occurring chelators such as humic acid may interfere with trace analysis such as synthetic hormones in environmental water samples. Hence, the effects of humic acid between 0.01 and 10 mg/L on the immunoassay were assessed. A loss of sensitivity was observed at higher humic acid concentrations, as shown in Fig. 2. Humic acid at 0.01 mg/L did not alter the sensitivity and color development of the assay. Humic acid above 0.01 mg/L, however, either inhibited enzyme activity or affected the antigen-antibody binding or both of these effects. At concentrations higher than 0.01 mg/L, loss of sigmoidal shape of the standard curve was observed. The extent of the decrease in assay sensitivity was proportional to the concentration of the humic acid. Similar results were also observed in the previous studies (Deng et al., 2003; Schneider et al., 2004, 2005). This may be due to EE2 forming a complex with the humic acid, making EE2 unavailable for antibody binding. As such, sample clean-up to remove interfering matrices to improve assay performance is highly recommended.

3.5. Assay Validation - Correlation between ELISA and GC/MS

The validation of the ELISA for the detection of EE2 residues was performed with the validated GC/MS method using the spiked water samples. Both ELISA and GC/MS presented good correlation with the spiking levels ranging from 10 to 50 ng/L, exhibiting a correlation coefficient (R^2) of 0.928 (Y = 0.84X – 0.14, n = 7, p-value = 0.0005) for ELISA and of 0.934 for GC/MS (Y = 0.89X – 1.95, n = 7, p-value = 0.0004). The ELISA correlated well with GC/MS analysis, exhibiting a correlation coefficient (R^2) of 0.934 (n = 7, p-value = 0.0004, Fig. 3). This suggests that the assay can be employed for reliable quantification of EE2. There was, however, a tendency for the ELISA to slightly overestimate the EE2 residues than GCMS, as shown in Fig. 3. The under-estimation by GC/MS was possibly due to incomplete derivatization which was required by the instrumental technique, hence showing slightly lower estimation, which was also reported in our previous study with the estrone analysis (Li et al., 2004).

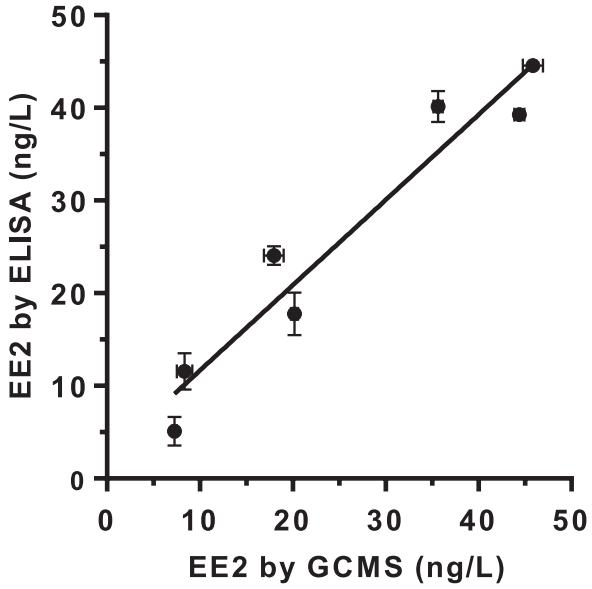


Fig. 3. Correlation between GC-MS analyses and ELISA results of the MilliQ water spiked with EE2. The linear regression equation is Y = 0.9203X + 2.45 (n = 7, R^2 = 0.934, P = 0.0004).

The % coefficient variation (%CV) of the ELISA and GC/MS data calculated from the spiked data ranged from 0.6% to 30% and 1 – 10%, respectively. The wider %CV of ELISA particularly at the lower concentrations suggested that special attention would be needed for low concentration analysis.

3.6. Environmental sample analysis

The developed assay was applied to an environmental study involving the monitoring of EE2/MeEE2 residues in river water collected from two catchments nearby the water treatment plant in Sydney, NSW. In total, 8 samples were analyzed by the developed assay using EE2 as the standard after the 800-fold enrichment using the SM2-Biobeads solid phase extraction was conducted. The EE2 levels in the two catchments were in low parts per trillion levels, but South Creek showed residues three times higher than Emigrant Creek (Fig. 4). The South Creek catchment is located about 50 km west of the Sydney central business district and forms the boundary between two big Western Sydney cities (Penrith and Blacktown). The Creek could be impacted by the discharges from the urban wastewater treatment plant (WWTP) and urban agricultural runoff. Moreover, South Creek is likely polluted by the sewage run-offs released from the three STPs in the area (St. Mary's, Riverstone and Quakers Hill). Samples from upstream of Riverstone STP and St. Mary STP showed the EE2/MeEE2 residues at 15 and 16 ng/L, and the EE2/MeEE2 residues from two downstream sites of South Creek were higher at 28 and 29 ng/L for Riverstone STP and St. Mary STP, respectively.

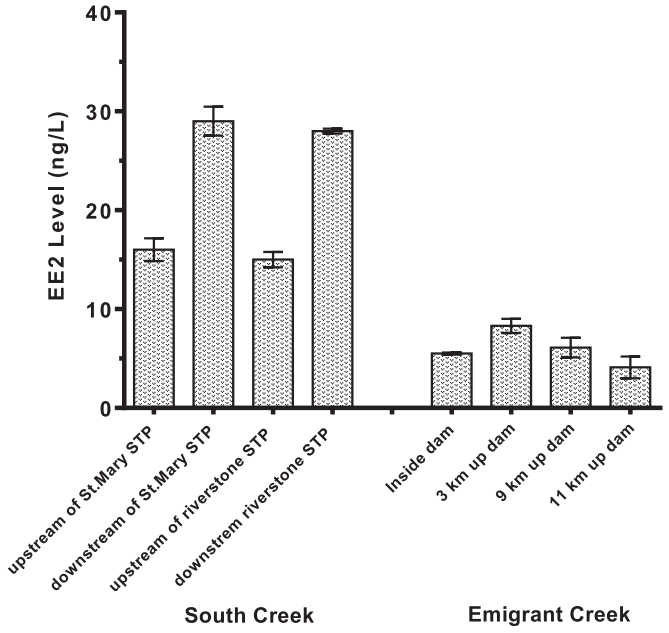


Fig. 4. The EE2 residues in the field water samples. The Creek water samples were collected upstream and downstream of the discharge point of effluent from the wastewater treatment plants in the two Creeks. Data are means of duplicate analysis ± SD (n = 8).

The Emigrant Creek catchment, on the other hand, is primarily used for horticulture (coffee, banana, stone fruit, and macadamia), plant nurseries and fish farms, grazing pasture for dairy cattle, and currently it is used to supplement the drinking water supply. The EE2/MeEE2 were detected at 5.5, 8.3, 6.1 and 4.1 ng/L inside the dam, 3 km up the dam, 9 km up dam and 11 km up the dam in Emigrant Creek locations, respectively. As reported by Pacakova et al. (2009) and Dorabawila and Gupta (2005), animal husbandry (agriculture) and urbanization are the likely contributors to the occurrence of estrogen in tributary or river water. Furthermore, run-off from cattle administered growth promoters, and sludge and manure applied to agricultural fields are the likely sources of synthetic hormone contamination at these sites. It is, therefore, reasonable to propose that higher EE2 levels found in South Creek are likely contributed from the urban activity nearby.

In this study, it was not reasonable to compare the results with a predicted no-effect concentration of EE2 (0.1 ng/L, (Young et al., 2004)), as the developed assay detected both EE2 and MeEE2 equally well and no further analyses were conducted to separate EE2 and MeEE2 estimates, although it is anticipated that most if not all of the residues are likely to be EE2 residues due to the in vivo metabolic activation. So we compared our results with the reported biologically significant levels (Andrew et al., 2008) as no regulatory levels are found in Australia. For example, vitellogenin (VTG) in male fish occurs at a concentration of EE2 as low as 11 ng/L (Humble et al., 2014), and a delay in the male gonad development and stimulation gonad maturation in both gender of zebra fish at a concentration of EE2 at 4 ng/L (Luzio et al., 2016a, 2016b).

The levels of EE2/MeEE2 in the South Creek were in the range of 15–29 ng/L. These are at the levels that could potentially affect the normal reproduction of fish. These results agreed well with Scott et al. (2014) where their study also showed EE2 levels of biological concern in river systems around Australia.

4. Conclusion

Highly specific polyclonal antibodies for the detection for EE2/MeEE2 were successfully raised against the immunogenic conjugate of EE2-acetate with the linker attachment at the C3 position. The heterologous hapten approach utilizing the Ab∝EE2-ACT1#4/ E2-ACT-HRP pair exhibited a good dose response curve with the LOD of 0.04 ± 0.02 μg/L and 0.04 ± 0.01 μg/L for EE2 and MeEE2 respectively. With the sample enrichment step with the SM2-Biobeads solid phase extraction, the assay exhibited the LOQ of 0.05 ± 0.01 ng/L for EE2/MeEE2 in water. Our hapten approach indeed resulted in the antibody highly specific to EE2 and MeEE2, exhibiting only negligible cross-reactivity for other steroidal sex hormones with various substituent groups at the C17-position (%CR < 3.1). Humic acid, the ionic strength of NaCl (salinity), and pH were among the predominant interfering factors of the assay. Adjusting pH to 7, and reducing salinity and humic acid to less than 0.01 M and 0.01 mg/L in water samples would be critical for quantification. The ELISA demonstrated acceptable precision and accuracy (as compared with the validated GC/MS) as a useful high-throughput screening tool. As such, ELISA will be a valuable analytical method for routine monitoring complementary to sophisticated instrumental techniques for detection of EE2 and MeEE2 residues in aquatic environments.

According to the developed ELISA, the EE2/MeEE2 levels in South Creek, where discharges from the urban WWTP is prevalent, may be sufficiently high to pose potential endocrine disrupting effects on aquatic habitats. Regular monitoring and systematic investigation to assess potential risks of EE2 residues posed on Sydney ecosystem need further and continual investigation and assessment. Additionally, despite our antibody exhibiting the desirable specificity as designed, the current assay still lacks the sensitivity needed for the parts per trillion analyses of EDCs without extensive sample enrichment. The sensitivity of the immunoassay will be further enhanced to improve end-user practicality for widespread use of the technique.

Acknowledgements

The authors gratefully acknowledge the financial support from the Australian Research Council Discovery Project (DP0559179). We are grateful for Miss C. S. H. Tan's assistance in hapten synthesis.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.06.077.

References

Adeel, M., Song, X., Wang, Y., Francis, D., Yang, Y., 2017. Environmental impact of estrogens on human, animal and plant life: a critical review. Environ. Int. 99, 107–119.

Andrew, M.N., Dunsta, R.H., O'Connor, W.A., Zwieten, L.V., Nixon, B., MacFarlane, G.R., 2008. Effects of 4-nonylphenol and 17α-ethynylestradiol exposure in the Sydney rock oyster, Saccostrea glomerata: vitellogenin induction and gonadal development. Aquat. Toxicol. 88, 39–47.

Avar, P., Zrínyi, Z., Maász, G., Takátsy, A., Lovas, S., G.-Tóth, L., Pirger, Z., 2016. β-Estradiol and ethinyl-estradiol contamination in the rivers of the Carpathian Basin. Environ. Sci. Pollut. Res. 23, 11630–11638.

Baronti, C., Curini, R., Ascenzo, G., Dicorcia, A., Gentil, A., Samperi, R., 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ. Sci. Technol. 34, 5059–5066.

Boenigk, J., Wiedlroither, A., Pfandl, K., 2005. Heavy metal toxicity and bioavailability of dissolved nutrients to bacterivorous flagellate are linked to suspended particles physical properties. Aquat. Toxicol. 71, 249–259.

Carvalho, A.R., Cardoso, V., Rodrigues, A., Benoliel, M.J., Duarte, E., 2016. Fate and Analysis of Endocrine-Disrupting Compounds in a Wastewater Treatment Plant in Portugal. Water Air Soil Pollut. 227. http://dx.doi.org/10.1007/s11270-016-2910-3.

Céspedes, R., Petrovic, M., Raldúa, D., Saura, Ú., Piña, B., Lacorte, S., Viana, P., Barceló, D., 2004. Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC–ESI-MS. Anal. Bioanal. Chem. 378, 697–708.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384.

Cunha, D.L., da Silva, S.M., Bila, D.M., Oliveira, J.L., Sarcinelli Pde, N., Larentis, A.L., 2016. Regulation of the synthetic estrogen 17α-ethinylestradiol in water bodies in Europe, the United States, and Brazil. Cad. Saude Publica 32. http://dx.doi.org/10.1590/0102-311X00056715.

Deng, A., Himmelsbach, M., Zhu, Q.Z., Frey, S., Sengl, M., Buchberger, W., 2003. Residue analysis of the pharmaceuticals diclofenac in different water types using ELISA and GC-MS. Environ. Sci. Technol. 37, 3422–3429.

Dorabawila, N., Gupta, G., 2005. Endocrine disrupter-estradiol-in Chesapeake Bay tributaries. J. Hazard Mater. 120, 67–71.
Gadd, J., Tremblay, L., Northcott, G., 2010. Steroid estrogens, conjugated estrogens and estrogenic activity in farm dairy shed effluents. Environ. Pollut. 158, 730–736.
Game, C., Gagnon, M., Webb, D., Lim, R., 2006. Endocrine disruption in male mosquitofish (Gambusia holbrooki) inhabiting wetlands in Western Australia. Ecotox 15, 665–672.
Gutendorf, B., Westendorf, J., 2001. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens. Toxico 166, 79–89.
Hintemann, T., Schneider, C., Schöler, H.F., Schneider, R.J., 2006. Field study using two immunoassays for the determination of estradiol and ethinylestradiol in the aquatic environment. Water Res. 40, 2287–2294.
Hirobe, M., Goda, Y., Okayasu, Y., Tomita, J., Takigami, H., Ike, M., Tanaka, H., 2006. The use of enzyme-linked immunosorbent assays (ELISA) for the determination of pollutants in environmental and industrial wastes. Water Sci. Technol. 54, 1–9.
Humble, J.L., Saaristo, M., Lindström, K., Lehtonen, K.K., Craft, J.A., 2014. Effects of 17α-ethinyl estradiol exposure on estrogen receptors α and β and vitellogenins A, B and C mRNA expression in the liver of sand goby (Pomatoschistus minutus). Mar. Environ. Res. 96, 12–18.
Lee, N.A., Kennedy, I.R., 2007. Food toxicants analysis: techniques, strategies and development. In: Yolanda, P. (Ed.), Immunoassay. Elsevier, pp. 91–146.
Li, C., Tyler, T., Zwieten, L.V., Lee, N.A., Kennedy, R.I., 2007. Optimisation of analytical method for estrogen in surface water and primary risk assessment in South Creek. Int. J. Water 3, 334–346.
Li, Z., Wang, S., Lee, N.A., Allan, R.D., Kennedy, I.R., 2004. Development of a solid-phase extraction-enzyme-linked immunosorbent assay method for the determination of estrone in water. Anal. Chim. Acta 503, 171–177.
Luzio, A., Matos, M., Santos, D., Fontaínhas-Fernandes, A.A., Monteiro, S.M., Coimbra, A.M., 2016a. Disruption of apoptosis pathways involved in zebrafish gonad differentiation by 17α-ethinylestradiol and fadrozole exposures. Aquat. Toxicol. 177, 269–284.
Luzio, A., Santos, D., Fontaínhas-Fernandes, A.A., Monteiro, S.M., Coimbra, A.M., 2016b. Effects of 17α-ethinylestradiol at different water temperatures on zebrafish sex differentiation and gonad development. Aquat. Toxicol. 174, 22–35.
Maltais, D., Roy, R.L., 2014. Effects of nonylphenol and ethinylestradiol on copper redhorse (Moxostoma hubbsi), an endangered species. Ecotoxicol. Environ. Saf. 108, 168–178.
Manickum, T., John, W., 2015. The current preference for the immuno-analytical ELISA method for quantitation of steroid hormones (endocrine disruptor compounds) in wastewater in South Africa. Anal. Bioanal. Chem. 407, 4949–4970.
Naldi, A.C., Fayad, P.B., Prévost, M., Sauvé, S., 2016. Analysis of steroid hormones and their conjugated forms in water and urine by on-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry. Chem. Cent. J. 10. http://dx.doi.org/10.1186/s13065-016-0174-z.
Ngamniyom, A., Silprasit, K., Panyarachun, B., 2012. Effects of pharmaceutical mestranol on estrogen receptor β mRNA expression levels and morphometry in the anal fins of adult Thai Rice fish (*Oryzias minutillus*). Kasetsart J. 46, 80–90.
Oberdorster, E., Cheek, A.O., 2001. Gender benders at the beach: endocrine disruption in marine and estuarine organisms. Environ. Toxicol. Chem. 20, 23–36.
Oh, S.M., Choung, S.Y., Sheen, Y.Y., Chung, K.H., 2000. Quantitative assessment of estrogenic activity in the water environment of Korea by the Escreen assay. Sci. Total Environ. 263, 161–169.
Pacakova, V., Loukotkova, L., Bosakova, Z., Stulik, K., 2009. Analysis for estrogen as environmental pollutants- A review. J. Sep. Sci. 32, 867–882.
Schneider, C., Sch¨oler, H.F., Schneider, R.J., 2005. Direct sub-ppt detection of the endocrine disruptor ethinylestradiol in water with a chemiluminescence enzyme-linked immunosorbent assay. Anal. Chim. Acta 551, 92–97.
Schneider, C., Schöler, H.F., Schneider, R.J., 2004. A novel enzyme-linked immunosorbent assay for ethynylestradiol using a long-chain biotinylated EE2 derivative. Steriods 69, 245–253.
Scott, P.D., Bartkow, M., Blockwell, S.J., Heather, M.C., Stuart, J.K., Richard, L., James, A.M., Helen, N., Dayanthi, N., Vincent, P., Louis, A.T., St.J.W, M., Frederic, D.L.L., 2014. An assessment of endrocrone ativity in Australian rivers using chemical and in vitro analyses. Environ. Sci. Pollut. Res 21, 12951–12967.
Senesi, N., Miano, T.M., 1994. Humic Substances in the Global Environment: Implications for Human Health. Elsevier, Armsterdam.
Ternes, T.A., Stumpg, M.M.J., Haberer, K., Wilken, R.D., Servos, M., 1999. Behavior and occurance of oestrogen in municipal sewage treatment plant-I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225, 81–90.
Vieira Dias, A.C., Gomes, F.W., Bila, D.M., Sant'Anna Jr, G.L., Dezotti, M., 2015. Analysis of estrogenic activity in environmental waters in Rio de Janeiro state (Brazil) using the yeast estrogen screen. Ecotoxicol. Environ. Saf. 120, 41–47.
Williams, M., Woods, M., Kumar, A., Ying, G.G., Shareef, A., Karkkainen, M., Kookana, R., 2007. In: CSIRO (Ed.), Endocrine Disrupting Chemicals in the Australian Riverine Environment. Braddon, ACT: Land and Water Australia, Australia.
Young, W.F., Whitehouse, P., Johnson, I., Sorokin, N., 2004. Proposed Predicted-no-effect-concentrations (PNECs) for Natural and Synthetic Steroid Oestrogens in Surface Waters. United Kingdom Environment Agency.
Zacs, D., Perkons, I., Bartkevics, V., 2016. Determination of steroidal oestrogens in tap water samples using solid-phase extraction on a molecularly imprinted polymer sorbent and quantification with gas chromatography-mass spectrometry (GC-MS). Environ. Monit. Assess. 188. http://dx.doi.org/10.1007/s10661-016-5435-8.