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17β-Estradiol residues and estrogenic activities in the Hawkesbury River, Australia

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

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ABSTRACT

Two highly sensitive ELISAs for the specific detection of 17β-estradiol (E2) residues were developed, showing the limits of detection (LOD, a concentration at 15% inhibition of color development) of 0.04 ± 0.02 μg/L and 0.05 ± 0.03 μg/L. The average recovery rate of the river water samples spiked with E2 at 1–50 ng/L range was 111.5% (68.6–252%) with the % relative standard deviation (RSD) of 0.5–86.3%. The ELISA demonstrated a good correlation with the GC-MS analyses of the spiked river water samples (r = 0.909). Applying the developed E2 ELISA assay to the monitoring of E2 residues in Hawkesbury River (New South Wales, Australia) found that all the tested creek samples contained E2 residues less than the biologically significant level of 10 ng/L. However, 25% of the water samples tested demonstrated the estrogen activity (determined by the yeast estrogen screening (YES) assay) above the levels that have been linked to the adverse effects in fish and other aquatic organisms (> 20 E2 Eq ng/L). It was apparent that the E2 residues together with the EE2 residues (reported in our previous study) contributed to most of the observed estrogenic activity in Hawkesbury River.

1. Introduction

A sustainable supply of safe drinking water from quality sources is a global challenge for the water industry, especially with a changing climate pattern. Water recycling is a long-term strategic plan for many countries, aiming to increase the amount of water that is recycled as part of managing precious drinking water resources. Inefficient operation of sewage treatment plants (STPs) may release undesirable endocrine disrupting compounds (EDCs) residues from pharmaceuticals and personal care products back to the aquatic ecosystem via the effluent run-off (Fox, 2001).

Among EDCs, steroidal estrogens are the most likely estrogenic candidates for the monitoring of treatment plant efficiencies. Natural or synthetic hormones used in oral contraceptive pills and hormone replacement therapy or used in animal husbandry have shown to found their ways to surface and groundwater (Goodman and Gilman, 1996; Arcand-Hoy et al., 1998). The concentrations of these estrogenic hormones in water sources are low, generally in parts per trillion levels, but their bio-activity could have considerable negative impacts on fetal and postnatal development in human and wildlife by altering their endocrine functions (Ying et al., 2002). One of the most potent estrogenic hormones is the natural steroid, 17β-estradiol (E2). E2 can bioaccumulate in fish, mainly in the bile, and also in both ovaries and testes of fish exposed to contaminated water (Gibson et al., 2005). Environmental analyses of E2 measured in the STP effluents revealed a concentration range of 0–150 ng/L (Wu et al., 2017; Barreiros et al., 2018).

Analysis of ultra-low levels of E2 in complex wastewater matrices still remains a great analytical challenge, despite advancements in the instrumentation of chromatography-mass spectrometry. Typically, the instrumental methodologies require an incorporation of time and labor intensive sample enrichment and clean-up steps. The requirement of expensive instrumentation and the technical expertise needed for reliable operation means access to such instrument could be beyond the reach of many developing countries, or even as routine analysis in developed countries such as Australia (Ying et al., 2002). Developing an alternative or complimentary analytical technique that features simplicity, cost-effectiveness, and rapid analytical throughputs, which complements the instrumental techniques, would be beneficial for long-term monitoring.

Enzyme-linked immunosorbent assay (ELISA) has significant advantages, which include simplicity, rapidity, high-sensitivity, reproducibility, and high selectivity. They are also effective for high-throughput

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

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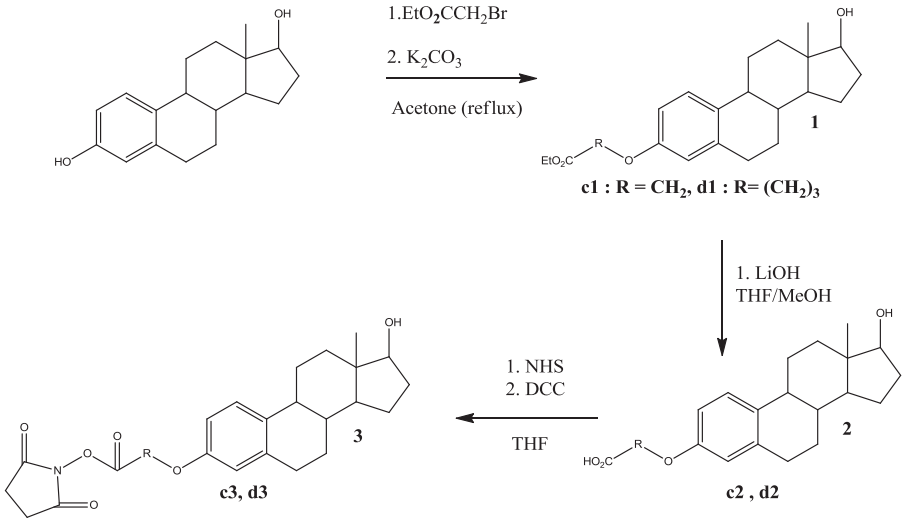

Fig. 1. A reaction scheme for the synthesis of 17β-estradiol (E2) haptens with the spacer arm attachment at the C3 hydroxyl group; **c 1–3** R = CH_2 (E2-acetate hapten), **d1-3** R = $(CH_2)_3$ (E2-butyrate hapten).

screening of large numbers of samples that impose a lower demand on labor costs. For the detection of steroidal estrogens in environmental samples by ELISA, it is important to consider cross-reactivity of structurally similar compounds (Huang and Sedlak, 2001; Uraipong et al., 2017).

Several environmental studies have been conducted using commercial estrogen ELISA kits intended for clinical uses without proper validation for environmental application. Thus, efforts have been made to develop ELISAs for environmental studies and compared with the commercial ELISA kits. For example, Goda et al. (2000) and Caron et al. (2010) compared their E2 ELISAs (with the LOD value of 0.1 μg/L for E2) with the commercial test kits. The specificity of their assays, however, did not match the commercial test kits and also did not meet criteria for analyzing complex wastewater samples containing structural similarities of estrogens (Caron et al., 2010). This has prompted us to develop an array of steroidal estrogens and androgen, particularly paying attention to their specificity for environmental analysis. These assays were aimed to assist with high-throughput analysis and risk assessment of EDCs in Australia (Li et al., 2004; Uraipong et al., 2013, 2017).

Our previous work on the EE2 using the sensitive and specific ELISA (Uraipong et al., 2017) found that the EE2 levels discharged from the urban wastewater treatment plant (WWTP) could pose potential endocrine disrupting effects on aquatic habitats. This paper describes the subsequent work on E2 as the most potent and prevalent estrogen in the aquatic environments with the development of a sensitive and E2 specific ELISA. It also includes the analysis of the estrogenic activity of the Hawkesbury River and other water resources that depended on all of the presenting estrogen residues. This is one of a very few studies conducted in New South Wales, Australia, that reported on the status of E2 and estrogenic activity of surface water.

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), dimethyl sulfoxide (DMSO), and dicyclohexylcarbodiimide (DCC), were purchased from either Aldrich (Sydney, Australia) or Sigma (Sydney, Australia). Dimethylformamide (DMF) and tetrahydrofuran (THF) are 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).

The yeast screening assay (YES) medium contains potassium phosphate monobasic anhydrous, ammonium sulfate, potassium hydroxide pellets, anhydrous magnesium sulfate, Iron (III) sulfate pentahydrate ($Fe_2(SO_4)_3$), L-leucine, free base L-histidine, free base adenine, hydrochloride L-arginine, L-methionine, L-tyrosine free base, L-isoleucine monohydrochloride, L-lysine, L-phenylalanine, inositol, L-glutamic acid free acid, L-valine, L-serine hydrochloride, thiamine hydrochloride, pyridoxine, *D*-pantothenic acid hemicalcium salt, d-biotin, anhydrous *D*-(+)-glucose anhydrous; mixed anomer, anhydrous copper (II) sulfate ($CuSO_4$), L-aspartic acid free acid, L-threonine, glycerol. Chlorophenol red-β-*D* galactopyranoside (CPRG) was supplied by Boehringer Mannheim (Lewes, East Sussex, UK), and 0.2 μm pore size filters were obtained from Whatman (Maidstone, Kent, UK)

2.2. Hapten synthesis

In order to achieve the desirable sensitivity and specificity, we investigated various heterologous systems, namely, hapten heterology and linker heterology with the linker attachment at C3, C6 or C17 positions. Of the seven haptens (Fig. 1.), five haptens were synthesized in-house, and two were from commercial sources. For the C3-linker attachment, 17β-estradiol acetate, 17β-estradiol-butyrate, 17α-ethinylestradiol-acetate, 17α-ethinylestradiol-butyrate were synthesized according to Uraipong et al. (2017). Estrone-3-hemisuccinate (E1-HS) consisting of the C3-linker was also prepared according to Li et al. (2004). For the C6- and C17-linker attachments, estradiol-one-6-*O*-carboxymethyl oxime (E2-CMO) (Sigma, St Louis, USA) and estradiol-3-hemisuccinate (E2-HS) (Sigma, St Louis, USA) were used respectively after the structures were confirmation by H^1-NMR.

2.3. Preparation of immunogens and enzyme conjugates

The immunogen was prepared by conjugating a carrier protein

(KLH) and a 17β-estradiol-3-acetate (denoted as E2-ACT-KLH). The competing hapten-enzyme conjugates were prepared by conjugating HRP with a series of haptenic analogs presented in Uraipong et al. (2017). 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 stand at 4 °C overnight. The conjugate solution was then dialyzed against phosphate buffered saline (PBS, pH 7.0) and the resultant solution was stored at 4 °C until use. Estradiol-one-6-*O*-carboxymethyl oxime (E2-CMO) (Sigma, St Louis, USA) and estradiol-3-hemisuccinate (E2-HS) (Sigma, St Louis, USA) 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

Two New Zealand white rabbits were immunized with E2-acetate-KLH and E2-butyrate-KLH. Briefly, the KLH conjugates were emulsified with an equal volume of Titer Max Gold adjuvant. The immunogen was injected subcutaneously in multiple sites into the rabbits. Follow-up the booster injections were given at 4-week intervals, blood was collected from the marginal ear vein 8–10 days after each booster injection. The serum was collected after centrifuging at 1500 × *g*.

2.5. Preparation of standard solution

Stock solutions of E2 at approximately 10 and 100 mg/L in anhydrous ethanol were prepared. To construct a calibration curve, a stock E2 standard solution was freshly prepared in glass tubes by diluting 1 in 100 from the 10 mg/L stock solution, giving a 100 μg/L standard solution (in 10% EtOH). Then the 100 μg/L standard solution was serially diluted 1 in 3 with 10% EtOH to obtain 33.3, 11.1, 3.3, 1.1, 0.37, 0.12, 0.041, and 0.014 μg/L working standards

2.6. Direct competitive ELISA

The purified anti-E2 antibodies were coated onto the microtiter plate as previously described. Then, the plate was washed three times with washing solution (0.05% Tween 20) and dried on an absorbent paper. The microtiters were incubated with 200 μL of 1% fish gelatine in PBS (FG/PBS) per well for 1 h at room temperature. The plate was again washed and dried as previously described. For the control, the wells were loaded with 100 μL of 10% methanol and 100 μL of enzyme conjugate. For the blank, the wells were loaded with 100 μL of 10% ethanol and 1% BSA/PBS (as an enzyme conjugate diluent). Standard solutions (100 μL per well) and an enzyme conjugate (100 μL per well) were applied to the respective wells and the mixture was incubated for 1 h at room temperature. Following the washing, 100 μL of TMB substrate solution was added to each well. The color reaction was developed for 30 min and stopped by adding 50 μL of 1.25 M H_2SO_4. The absorbance was measured at 450 nm using a microplate reader.

2.7. Cross-reactivity

The specificity of the ELISA was evaluated using structurally related E2 analogs selected to provide information on their relative sensitivity. These were 17α-ethinylestradiol, estriol, estrone, estradiol dipropionate, progesterone, 17α-estradiol, medroxyprogesterone, ethynylestradiol-3-methyl ether and 17α-ethynylestradiol-3-cyclopentyl ether, which may be found in wastewater. Percent cross-reactivity (%CR) was calculated at IC_{50} values (a midpoint in the standard curve).

$$\text{Cross reactivity}(\%)\text{at}IC_{50} = \left(\frac{IC_{50}(\text{E2})}{IC_{50}(\text{testcompound})}\right)\times 100$$

2.8. Field water samples and the sample preparation

Water samples were collected from four main locations in New South Wales (NSW) and Victoria (VIC), Australia, including South Creek in Sydney Basin, Emigrant Creek at Ballina, NSW, Wilsons River at Lismore, NSW, and in the Murray River at Albury-Wodonga at the boundary of NSW and VIC. All water samples were extracted and concentrated using SM2-Biobeads solid phase extraction as follows. Briefly, 8 L of the sample was extracted using the SM2-Biobeads solid phase extraction method to a final volume of 10 mL in dichloromethane (DCM). A 2 mL aliquot was dried with N_2 at 60 °C, and re-dissolved in 1 mL of 10% methanol (Uraipong et al., 2017).

2.9. Validation of ELISA with GC/MS using E2 spiked field water samples

The sample preparation and analysis of 17α-estradiol by GC-MS were conducted according to Li et al. (2007). The river sample collected from South Creek in the Sydney Basin was used in the correlation studies with GC-MS. The river sample (8 L) was spiked with E1, E2, EE2 and E3 at 0–50 ng/L. All water samples were extracted and concentrated using SM2-Biobeads solid phase extraction. Samples (8 L) were extracted and concentrated to a final volume of 10 mL in dichloromethane (DCM). These were subsequently reduced to dryness under N_2 at 60 °C prior to dissolution in 1 mL of 10% ethanol for immunoassay. GC-MS and ELISA analysis of the spiked samples were performed independently in the separate laboratories using different E2 standards.

2.10. Recombinant yeast estrogen screen (YES) assay

Protocol and the recombinant yeast was a generous gift of Prof. J.P. Sumpter, Brunel University, UK. The recombinant yeast estrogen screen (YES) bioassay for estrogenic activity measurement (including details of medium components) previously described by Routledge and Sumpter (1997) were conducted on the creek water samples. All yeast bioassays were carried out in a Type II laminar flow cabinet to minimize aerosol formation. Each assay plate was assayed with at least triplicate sample wells, at least one row of blanks (200 μL assay medium only), and an E2 calibration curve (0.02–100 μg/L). A stock solution of each test steroidal hormone in ethanol was serially diluted in ethanol. Aliquots of 10 μL of the respective test hormone solution were then transferred to a sterile microtiter plate and the ethanol was allowed to evaporate at room temperature. Aliquots of 200 μL per well of the seeded assay medium containing CPRG and the recombinant yeast were then added to all testing wells. Calibration curves of E2 as a positive control was included in every yeast screen assay. The plates were sealed with an autoclave tape and it was shaken thoroughly for 2 min on a microtitre plate shaker prior to incubation at 32 °C in a naturally ventilated incubator. The plates were shaken vigorously on the plate shaker for 2 min after 24 and 48 h, to disperse the growing cells. On the fourth day, after incubating for 72 h (3 days), the plate was shaken for 2 min, left to stand for 1 h to allow the yeast to settle, and then the absorbance was read at 570 nm and 620 nm (for turbidity).

2.11. Data analysis and statistical evaluation

All experiments were conducted at least in duplicates ($n \geq 2$) and the results were presented as a mean ± standard deviation. Non-linear regression followed by the four-parameter logistic analysis and correlation were performed using GraphPad Prism version 7.02 for Windows, GraphPad Software, La Jolla California USA.

3. Results and discussions

3.1. Hapten synthesis and selection for the competitive immunoassays

An ideal hapten is one that preserves as closely as possible the structure of the target analyte in size, shape (3D) and electronic properties for specific antibody production (Szurdoki et al., 1995). Examining the backbone structure of E2 provided three possible linker attachment positions at C3, C6, and C17, which could give rise to different binding specificity. Of these possibilities, the C6-linker synthesized derivatives were thought to provide better preservation of the estrogen structures. Due to the orientation of the hapten allowing both C3- and C17- hydroxyl groups to be exposed to the immune response, the assays may exhibit significant cross-reactivity with other estrogens and possibly their sulfate and glucuronide metabolites. Attaching a linker at the C17 position has been rarely considered for raising E2 specific antibodies. This is because antibodies raised are likely to be more specific to the C3 moiety, which is common to most of the estrogens. Our rationale for selecting the C3-linker attachment as the most appropriate hapten is that it allows the C17 and C18 substituent groups to be distal from the point of attachment to a carrier protein. Antibodies raised against this hapten (i.e., linker attached at C3) will differentiate 17β-estradiol from other steroids with a similar backbone structure, as shown in our previous work with E1 (Li et al., 2004; Uraipong et al., 2017).

Hence, two hapten molecules based on E2 for raising specific antibodies were synthesized; one with an acetate linker with three-carbon chain for better solubility in aqueous solutions, and the other with a butyrate linker with a four-carbon chain. Both linkers consisted of saturated carbon bonds to ensure sufficient flexibility after they were conjugated to a carrier protein, but not long enough to fold into the carrier protein. The saturated carbon chain was chosen to minimize the undesirable immune recognition of the linker. The hapten with a butyrate linker was less soluble in an aqueous solution than the same hapten with a shorter linker. The conjugation of the butyrate hapten performed in an aqueous solution even with a small amount of dimethylformamide did not yield the expected epitope density. Commercial haptens, E2-CMO and E2-HS, with the linker attachment at the C6 and C17 positions were also evaluated for their antibody sensitivity and specificity. To investigate the effects of hapten heterology, EE2-acetate and EE2-butyrate haptens were also synthesized to be used as competing haptens (Fig. 1).

3.2. Assay sensitivity

Evidently, all the haptens were highly immunogenic resulting in eight antibodies (two for each immunizing hapten) with high titers against their respective immunizing haptens. The serum titers improved notably with the subsequent booster injections, showing high immunoreactivity of the haptens and affinity maturation of the raised antibodies. To achieve the best sensitivity, the hapten heterology, and linker attachment heterology was employed to manipulate the antibody binding affinity between the competing hapten and the free E2 in a competitive immunoassay. Hence, the optimum condition of each direct competitive assay was determined by titrating the seven enzyme conjugates (EE2-acetate-HRP, EE2-butyrate-HRP, E2-acetate-HRP, E2-butyrate-HRP, E2-CMO-HRP, E2-HS-HRP, and E1-HS-HRP) against each of the eight selected antibodies (Abα E2-CMO, Abα E2-HS, Abα E2-ACT1#3, Abα E2-ACT2#3, Abα E2-BUT1#3 and Abα E2-BUT2#3). For the purpose of comparison between assays, the sensitivity is expressed by IC_{50} values (the concentration of analytes producing 50% of inhibition), the lower IC_{50} values, the higher the sensitivity. The results are summarized in Table 1.

The antibodies raised against E2-HS-KLH (Abα E2-HS) with the C17-linker attachment poorly recognized the haptens with the C3-linker attachments, probably due to not enough sharing structures between these two haptens. The Abα E2-HS, however, recognized the E2-CMO hapten with the C6-linker attachment better since these two haptens had common C3 moiety. As a result, Abα E2-HS together with E2-CMO-HRP and E2-HS-HRP gave the better assay sensitivity for E2. The best sensitivity was given by the heterologous assay with an IC_{50} value of 1.1 ± 0.13 μg/L. The antibodies raised against E2-CMO-KLH with the C6-linker attachment showed an interesting result. The best sensitivity was given by the homologous assay using the same hapten as a competitor. All of the heterologous assays produced poorer sensitivity, except for the assay using EE2-acetate-HRP, which rivaled the homologous assay. It is thought that the poor sensitivity of the heterologous assay using EE2-butyrate-HRP could be due to poor conjugation of the hydrophobic hapten to the enzyme. Despite the poor solubility and conjugation, Abα E2-BUT did show good binding responses with all the competing haptens, except for E2-CMO, perhaps for the same reason mentioned above. Despite being raised against the same hapten, the two Abα E2-BUT pair and the two Abα E2-ACT pair showed different specificity. This is evidenced by their sensitivity against different competing haptens, especially against the haptens with different linker attachment points (e.g., E2-CMO, E2-HS). From the results, it can be deduced that the Abα E2-BUT2 and Abα E2-ACT2 may be more specific to the C17 moiety. Whereas Abα E2-BUT1 and Abα E2-ACT1 may have broader binding moieties covering C17 and some common backbone.

Table 1
The IC_{50} values of the optimized calibration curves of the ELISA using Abα E2-ACT1#3 and Abα E2-BUT1#3 against seven different hapten-enzyme conjugates.

Enzyme conjugates	IC_{50} (μg/L)	
	Abα E2-ACT1#3	Abα E2-BUT1#3
17α-ethinylestradiol-acetate (EE2-ACT-HRP)	0.4 ± 0.02	0.5 ± 0.03
17α-ethinylestradiol-butyrate (EE2-BUT-HRP)	0.6 ± 0.1	0.8 ± 0.1
17β-estradiol-acetate (E2-ACT-HRP)	0.6 ± 0.1	0.8 ± 0.2
17β-estradiol-butyrate (E2-BUT-HRP)	2.4 ± 0.3	6.3 ± 0.03
17β-estradiol-hemisuccinate (E2-HS-HRP)	1.9 ± 0.2	1.7 ± 0.01
Estrone-3-hemisuccinate (E1-HS-HRP)	2.9 ± 0.1	1.9 ± 0.01
17β-estradiol-one6-*O*-carboxymethyl-oxime (E2-CMO-HRP)	7.7 ± 0.4	ND*

ND* = Not detectable.

The Abα E2-ACT1 and Abα E2-BUT1 generally showed good responses with most if not all of the seven enzyme conjugates. The poorest response of the Abα E2-ACT1 was with E2-CMO. Both Abα E2-BUT1 and Abα E2-BUT1 responded poorly with E2-CMO-HRP as well. The hapten heterologous assays, for example, Abα E2-ACT against EE2-acetate-HRP or EE2-butyrate-HRP, displayed superior sensitivity. Of the different heterologous systems investigated, hapten heterology gave the greatest influence on the assay sensitivity. For example, the heterologous assay using the Abα E2-ACT1#3/ EE2-acetate-HRP pair gave higher sensitivity than its counterpart homologous assay using the Abα E2-ACT1#3/ E2-acetate-HRP. Similarly, the heterologous assay based on the Abα E2-BUT1#3/ E2-ACT-HRP gave seven-time higher sensitivity (based on the IC_{50} values) than its counterpart homologous assay Abα E2-BUT1#3/ EE2-BUT-HRP. Interesting, all of the antibodies did not respond as well with E1-HS hapten, as EE2-ACT or EE2-BUT hapten, EE2 seems to have a greater structural variation at C17 than E1. The best sensitivity was given by the Abα E2-ACT1#3/ EE2-acetate-HRP pair, with an IC_{50} value of 0.38 ± 0.07 μg/L and the limit of detection (LOD) of 0.04 ± 0.01 μg/L (Fig. 2). This assay also gave good color development and lower variability between assays.

Assessing the antibody responses to different competing haptens suggests that haptens with C6-linker attachment may not be as versatile as haptens with other points of linker attachment. Of the three points of

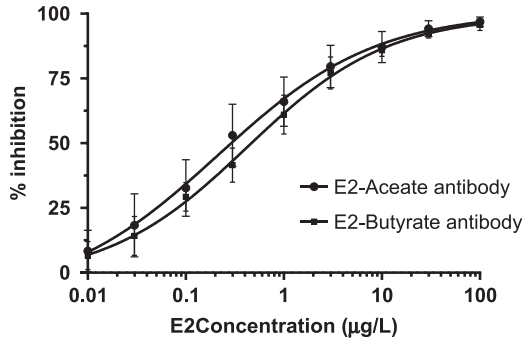

Fig. 2. Standard curves of the AbαE2-ACT1#3/ EE2-acetate-HRP pair and AbαE2-BUT1#3/ EE2-acetate-HRP pair. Each data point is an average of 11 analyses run on different days.

linker attachment evaluated, the C3-linker attachment seems to provide high-affinity antibodies and good responses with a competing hapten in a competitive assay. Given that the hapten synthesis of C3-linker attachment is not as demanding as that of C6-linker attachment and with good yields, the C3-linker attachment is recommended for developing E2 immunoassay.

3.3. Assay specificity

Two best antibodies from each immunizing hapten, AbαE2-ACT1#3 and AbαE2-BUT1#3, were selected for the specificity study. Both antibodies showed only negligible cross-reactivity (< 2.5%) with the nine structurally-related compounds, indicating the high specificity for E2. The cross-reactivity of estriol was slightly higher than those of the other test compounds: 2.4% in AbαE2-ACT1#3/EE2-acetate12-HRP pair, 0.7% in AbαE2-BUT 1#3/EE2-aceate20-HRP pair. Estradiol dipropionate also presented slight but notable cross-reactivity of 1.4% in the AbαE2-BUT 1#3/EE2-aceate20-HRP pair (Table 2). Overall, the cross-reactivity of the E2 ELISAs for the typical estrogenic metabolites was lower than previously reported studies that presented > 2.5% cross-reactivity for the same compounds (Goda et al., 2000; Hintemann et al., 2006). Evidently, the haptens with a C3 linker were able to provide the necessary structural orientation to elicit antibodies with the binding preference to C17 substituent groups. It is most probable that C17-hydroxyl group is buried in the hydrophobic pockets of the binding site, as shown in the crystallography of an E2-antibody-E2 complex by Monnet et al. (2002). The E2-ACT and E2-BUT haptens, though differing only in the length of the linker, elicited antibodies with slightly different binding positions, reflected by their relative cross-reactivity for estriol and estradiol dipropionate. Nevertheless, both antibodies are considered highly specific to E2 and useful bioaffinity molecules for developing highly specific ELISA for environmental application.

Table 3
% Recoveries of the spiked E2 by GC-MS and ELISA (n = 2).

Spike (ng L^{-1})	GC-MS		ELISA	
	Average Rec (%)	SD Rec (%)	Average Rec (%)	SD Rec (%)
1	112.0	15.6	130.0	21.2
1	134.0	49.5	252.0	29.7
1	119.0	15.6	140.0	86.3
10	53.7	31.3	108.0	11.2
10	128.3	24.1	95.0	7.1
10	124.6	0.1	93.6	14.1
10	91.5	33.5	76.2	6.9
12.5	92.8	4.7	133.3	7.4
16	99.3	1.1	98.8	8.0
20	97.4	1.6	103.8	5.2
20	96.7	2.5	116.3	21.6
20	73.2	8.9	115.7	19.0
20	83.3	2.2	94.8	1.2
25	103.4	2.3	99.6	2.5
50	88.7	1.8	98.8	3.6
50	68.6	2.3	70.3	4.0
50	86.0	5.1	68.6	0.5

Regression equations: GC-MS = 0.794 *X(spiked) + 1.722 (n = 17, R^2 = 0.9507); ELISA = 0.767 *X (spiked) + 3.208 (n = 17, R^2 = 0.908). Average Rec (%) = average % recovery.
SD Rec (%) = standard deviation of % recovery.

3.4. Accuracy and precision

The accuracy of E2 ELISA was evaluated by comparing results with those obtained from GC-MS. The E2 was spiked in a range between 0 and 50 ng/L into the river water collected from Hawkesbury river away from the sewage treatment plant. The spiked samples were extracted and concentrated by the SM2-Biobeads solid phase extraction. Prior to the study, the river water sample was analyzed by GC-MS to confirm the background E2 residues, which were accounted for in the calculation of the recoveries. The analysis of the spiked samples by GC-MS and ELISA were performed independently by different teams at different locations, and the data were collated for the statistical analysis.

The GC-MS on average gave 97.2% recovery (53.7–134%) and the ELISA gave 115.5% (68.6–252%) (Table 3). Higher recoveries were obtained at lower concentrations up to 25 ng L^{-1} and beyond this concentration, the % recovery reduced for both techniques. The ELISA showed comparable precision as GC-MS in this spike study, evidenced by the similar average relative standard deviation (RSD); 12.7% (0.1–58.2%) for the GC-MS and 11.7% (0.7–61.6%) for the ELISA (Table 3). Despite the two techniques were performed independently using different E2 standard solutions, the E2 ELISA results were well correlated with those of GC-MS, with a correlation coefficient (r^2) of 0.909. There was, however, a slight tendency for the ELISA values to be slightly higher than GC-MS values (Fig. 3).

Table 2
The IC_{50} values and % cross-reactivity for selected estrogens with the two E2 ELISAs.

Compounds	AbαE2-ACT1#3/EE2-acetate-HRP			AbαE2-BUT1#3/EE2-acetate-HRP		
	IC_{50} (µg/L)	IC_{50} (mol/L)	CR (%)	IC_{50} (µg /L)	IC_{50} (mol/L)	CR (%)
17β- estradiol	0.4	1.5×10^{-9}	100	0.5	1.8×10^{-9}	100
17α-ethinylestradiol	> 100	$> 3.4 \times 10^{-7}$	< 0.4	> 100	$> 3.4 \times 10^{-7}$	< 0.5
Estriol	16.8	5.8×10^{-8}	2.5	75.7	2.6×10^{-8}	0.7
Estrone	> 100	$> 3.7 \times 10^{-7}$	< 0.4	> 100	$> 3.7 \times 10^{-7}$	< 0.5
Estradiol dipropionate	> 100	$> 2.6 \times 10^{-7}$	< 0.6	36.5	9.5×10^{-8}	1.9
Progesterone	> 100	$> 3.2 \times 10^{-7}$	< 0.5	> 100	$> 3.2 \times 10^{-7}$	< 0.6
17α- estradiol	> 100	$> 3.7 \times 10^{-7}$	< 0.4	> 100	$> 3.7 \times 10^{-7}$	< 0.5
Medroxyprogesterone	> 100	$> 2.9 \times 10^{-7}$	< 0.5	> 100	$> 2.9 \times 10^{-7}$	< 0.6
Ethinylestradiol-3- methyl ether (Mestranol)	> 100	$> 3.2 \times 10^{-7}$	< 0.5	> 100	$> 3.2 \times 10^{-7}$	< 0.6
17α -ethinylestradiol 3-cyclopentyl ether (quinestrol)	> 100	$> 2.7 \times 10^{-7}$	< 0.5	> 100	$> 2.7 \times 10^{-7}$	< 0.7

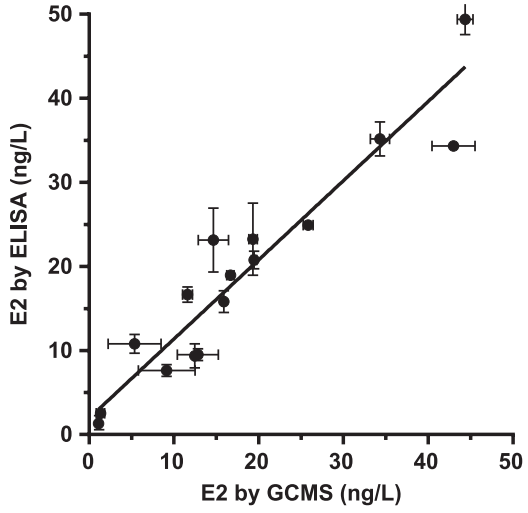

Fig. 3. Correlation between GC-MS analyses and E2 ELISA results of the river water samples spiked with E2. The linear regression equation is Y = (0.942 ± 0.077) X + (1.941 ± 1.639) (n = 17, R^2 = 0.909, P = < 0.0001).

3.5. Survey of E2 residues in South Creek and Emigrant Creek, Hawkesbury River

The developed E2 ELISA assay was applied to the environmental study of E2 residues in Hawkesbury River (Sydney regions, New South Wales) particularly targeted South Creek and Emigrant Creek, which located nearby the water treatment plants. The South Creek catchment is likely to be impacted by the discharges from the urban wastewater treatment plant (WWTP) and urban agricultural runoff from two large western Sydney suburbs (Penrith and Blacktown) and the water in Emigrant Creek comes from a catchment that is an agricultural area with rural residential and some tourist development. The E2 residues compared with EE2 residues in each collection points are shown in Fig. 4.

The E2 levels in both catchments were in low parts per trillion levels. The E2 levels in South Creek were 8.5 times higher than Emigrant Creek on an average. The results are in the agreement with our previous study on the EE2 levels in these two Creeks (Uraipong et al., 2017). The EE2 levels were higher than the E2 levels, which were 7 and 17 times more in South Creek and Emigrant Creek, respectively. This could be due to a greater resistance of EE2 to the degradation and its greater tendency to absorb onto organic matter, accumulate in sediment, and

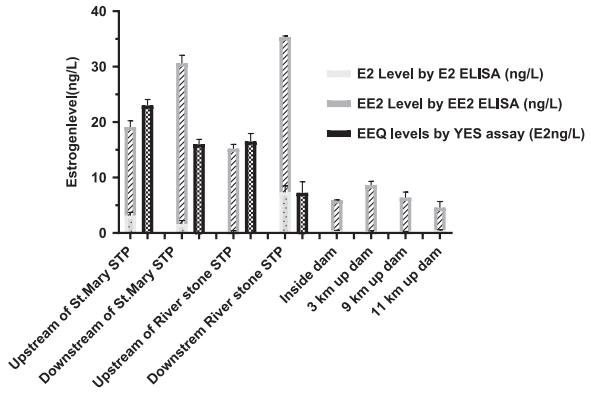

Fig. 4. 17α-Estradiol (E2), 17α-ethinylestradiol (EE2), and estrogenic activity expressed as E2 equivalent (EEQ) values levels in field samples collected in South Creek and Emigrant Creek. Creek water samples were collected upstream and downstream of the discharge point of effluent from wastewater treatment plants.

concentrate in biota (Aris et al., 2014) (Fig. 4). The E2 levels in Emigrant Creek were generally less than 0.5 ng/L. The inside dam and 3 km up dam showed the E2 level at 0.35 and 0.30 ng/L respectively. The sample from 9 km upstream of the dam in Emigrant Creek had a slightly lower E2 level at 0.27 ng/L, while 11 km upstream of the dam had the highest E2 level at 0.48 ng/L.

The in upstream of Riverstone STP and St. Mary STP South Creek showed the E2 levels at 0.2 and 3.1 ng/L respectively. Two downstream sites of South Creek had higher levels at 7.3 and 1.6 ng/L for Riverstone STP and St. Mary STP, respectively. The sources of E2 residues were likely from both the agricultural activity and urbanization because the South Creek catchment is in close proximity to the Sydney CBD (only about 50 km west of the Sydney CBD). Notably, the E2 levels from South Creek catchment were lower than the EE2 levels we reported previously (Uraipong et al., 2017). However, there was no obvious relationship between the E2 and EE2 residues, or the locations of the samples relative to STPs in this study. It was notable, however, that urbanization seemed to be a contributor to the hormone load in the environment, evidenced by the significantly higher EE2 levels compared to the E2 levels (> 84% of the summative estrogen residues) and higher estrogen levels in South Creek than those in Emigrant Creek (Fig. 4).

The E2 levels in South Creek were similar to the other Australian studies conducted in the last decade, which showed the concentrations below 6.4 ng/L (Williams et al., 2007). For example, the E2 residues in the discharge of Victorian WWTPs were approximately 5 ng/L (Mispagel et al., 2009). The E2 levels in WWTPS in southern Queensland were 4.7–5 ng/L (Chapman, 2003; Khan et al., 2004). Our results, as well as the previous studies, suggest that the E2 levels in Australian water resource are likely to be below the biologically significant levels of 10 ng/L (Czarny et al., 2017).

3.6. Estrogenic activity of the water samples

Water samples collected from South Creek were subjected to the YES assay to estimate the estrogenic activity expressed as 7β-estradiol equivalent (EEQ) values. The YES assay was not performed on Emigrant Creek samples due to insufficient quantities. The total EEQ levels of the South Creek water samples ranged from 7.1 and 23.0 ng/L. The water in the upstream of South Creek mainly comes from the runoff into the catchment and the EEQ levels for these sites were 23.0 and 13.9 ng/L. At the downstream discharge point of St. Mary STP and further down from STP, the EEQ levels were 16.0 and 13.6 ng/L, respectively. The EEQ level of the immediate downstream of Riverstone STP was 7.3 ng/L, whereas the EEQ level of the site, which is located upstream of Riverstone STP, but downstream of Quakers Hill STP, was 16.5 ng/L. Furthermore, the EEQ levels downstream in South Creek represented the total EDC levels brought into the Hawkesbury River, which was measured at 7.1 ng/L, also showing an overall low estrogenic activity. It was noted that the EEQ levels shown in Fig. 4 were generally lower than the summative levels of E2 (Section 3.5) and EE2 residues (Uraipong et al., 2017). This suggests that E2 and EE2 are the main residues contributing to the estrogenic activity shown by the YES assay.

Two locations had the EEQs values less than 10 ng/L, 4 locations had the EEQs values between 10 and 20 ng/L, and only one location had the EEQs value higher than 20 ng/L. Thus, All of the creek samples (95%) showed estrogenic activities of less than 100 ng/L, which are in good agreement with Scott et al. (2014). These results, however, were higher than the previous studies; for example, the WWTP effluents in South Australia showing 7.9 ng/L EEQ, and 8–16 ng/L EEQ in Victoria, (Mispagel et al., 2005, 2009) and < 1–4.2 ng/L EEQ in the STPs in south Queensland (Leusch et al., 2006). Meanwhile, these results were near the lower end of the range observed in several overseas studies; for example, the effluents in Japan (5–15 ng/L EEQ), the USA (44–151 ng/L EEQ), Sweden (< 0.1–15 ng/L EEQ) and Switzerland (0.1–90 ng/L EEQ) (Matsui et al., 2000; Tilton et al., 2002; Svenson et al., 2003;

Rutishauser et al., 2004).

4. Conclusion

Two highly sensitive and specific competitive ELISAs for the detection of E2 in aquatic environments were successfully developed and applied to the survey of E2 residues in Hawkesbury River, NSW. Using the E2 haptens synthesized with the linkers attached at the C3- hydroxyl group, the polyclonal antibodies that are highly sensitive and specific to E2 were developed. The spike and recovery study and the correlation with GC-MS indicated good accuracy (R^2 = 0.909). The survey of the E2 residues in South Creek and Emigrant Creeks in Hawkesbury river using the E2-acetate ELISA, identified the locations with detectable E2 in surface water. In general, all the creek samples contained the E2 residues less than the biologically significant level of 10 ng/L. However, 25% of the water samples tested demonstrated the estrogen activity (as EEQ determined by YES assay) above the levels that have been linked to the adverse effects in fish and other aquatic organisms (> 20 E2 Eq ng/L). The E2 levels upstream compared to those downstream from the discharge point did not appreciably increase the estrogenic potencies to a significant level. The EEQ values were lower than the summative concentrations of E2 and EE2, suggesting that the E2 and EE2 were likely the major contributors to the observed estrogenic activity. In summary, this study demonstrated that the combination of ELISA and YES assay clearly has applicability as analytical tools for water quality monitoring and toxicological assessment of EDCs.

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