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Supplemental Materials

In vitro and *in vivo* Estrogenic Effects of 17α-Estadiol in Medaka (*Oryzias latipes*)

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These supplemental materials provide detailed descriptions of yeast two-hybrid assay for estrogenic agonist activity, concentration determination of 17α-E2 and 17β-E2 in exposure water samples by LC-ESI-MS/MS analysis and environmental concentration levels of 17α-E2 reported in different environmental matrices in recent years.

1. Yeast two-hybrid assay for estrogenic agonist activity

The yeast cells were preincubated at 30°C for 16 hours in 2 ml medium (6.7 g L^{-1} Difco yeast nitrogen base without amino acids, 0.2% glucose, 300 mg L^{-1} L-isoleucine, 1500 mg L^{-1} L-valine, 200 mg L^{-1} L-adenine hemisulfate salt, 200 mg L^{-1} L-arginine HCl, 200 mg L^{-1} L-histidine HCl monohydrate, 300 mg L^{-1} L-lysine HCl, 200mg l^{-1} L-methionine, 500mg L^{-1} L-phenylalanine, 200 mg L^{-1} L-threonine, 300 mg L^{-1} L-tyrosine, 200 mg L^{-1} L-uracil (Sigma, USA)). 50 μL of overnight culture and 2.5 μL of DMSO solution containing test chemical were then added to 200 μL of fresh medium (2% galactose) in a microtube (Axygen Scientific, U.S.A.), respectively. After yeasts were cultured for 4 h at 30°C, 150 μL of the above culture was fractionated, and its absorbance at 595 nm was detected. The residual culture (100 μL) was centrifuged at 4 ºC (15000 rpm) for 5 min, and the collected cells were resuspended in 200 μL of Z buffer (0.1 M sodium phosphate (pH = 7.0), 10 mM KCl, 1 mM $MgSO_4$) containing 1mg mL^{-1} Zymolyase 20T (Seikagaku, Tokyo), and incubated for 20 min at 30ºC. The enzymatic reaction was started by the addition of 40 μL of 4 mg mL^{-1} 2-nitrophenyl-β-D-galactopyranoside (ONPG, Tokyo Kasei, Tokyo, Japan), and incubated for 20 min at 30ºC. Then the enzymatic reaction was stopped by adding 1 M Na_2CO_3 (100 μL). After the above solution was centrifuged, 150 μL aliquots were placed into 96 wells of a microplate. Absorbances at 415 and 570 nm were read on a microplate reader (Bio RAD 550, USA), and the β-galactosidase activity (U) was calculated according to Equation :

$$U=1000\times([OD_{415}]-[1.75\times OD_{570}]/([t]\times[v]\times[OD_{595}])$$

where t represents the reaction time (min); v is the volume of the culture used in the assay (ml); OD_{595} is the cell density at the start of the assay; OD_{415} is the absorbance by

o-nitrophenol at the end of the reaction, and OD_{570} is the light scattering at the end of the reaction.

2. Analysis of actual estrogens in exposure tanks

2. 1 Sample preparation

The water samples (2 L for vehicle control groups, 0.1 ng $L^{-1}$and 1 ng L^{-1} exposure groups; 500 mL for 10 ng L^{-1} and 100 ng L^{-1} exposure groups; 10 mL for 1000 ng $L^{-1}$and 10000 ng L^{-1} exposure groups) were spiked with 10 ng of 17β-E2-d_3 and were extracted through the HLB cartridges (6 mL, 500 mg, Waters, Milford, MA, USA) at a flow rate of 5-10 mL/min. The cartridge was preconditioned with 6 mL of ethyl acetate, 6 mL of acetonitrile and 12 mL of distilled water. Then the cartridges were rinsed with 10 mL of distilled water and dried under a flow of nitrogen. Target analytes (17α-E2 and 17β-E2) were subsequently eluted with 15 mL of ethyl acetate. The eluates were evaporated to dryness under a gentle stream of nitrogen and reconstituted with 0.5 mL of methonal for LC-ESI-MS/MS analysis.

2. 2 LC-ESI-MS/MS analysis

The LC apparatus was an Acquity Ultra Performance LC (Waters, USA). Acquity UPLC® BEH C8 column (100 × 2.1 mm, 1.7 μm particle size) (Waters, USA) was used for separation. The column was maintained at 40°C at a flow rate of 0.3 mL/min and the injection volume was 5 μL. Methanol and ultrapure water were used as mobile phases. Methanol was initially increased linearly from 10% to 50% in 0.5 min, to 80% in the next 5.5 min, to 100% in the following 1.0 min, and kept for 1.0 min. The column was then equilibrated for 3.0 min.

Mass spectrometry was performed using a Quattro Premier™ XE detector (Waters, USA) which was operated with ESI in the negative ion (NI) mode. The detection conditions of the mass spectrometer were as follows: capillary voltage, 3.0 kV; source temperature, 110°C;

desolvation temperature, 400°C; desolvation gas flow, 800 L/h; and cone gas flow, 50 L/h. Finally, the data acquisition was performed under time-segmented conditions based on the chromatographic separation of the target compounds to maximize sensitivity of detection (Table 1).

Table S1. Parameters for analyze estrogens by LC-ESI-MS/MS

Compound	MRM transition	Cone voltage (V)	Collision energy (eV)
17α-estradiol (17α-E2)	271 > 145	60	36
	271 > 183		42
17β-estradiol (17β-E2)	271 > 145	60	48
	271 > 183		38
17β-estradiol-d_3 (17β-E2-d_3)	274 > 185	58	46

2. 3 Quality assurance and Quality control

Identification of the target estrogens was accomplished by comparing the retention time (within 2%) and the ratio (within 20%) of the two selected precursor ion-production ion transition with those of standards. To automatically correct the losses of analytes during sample preparation and the matrix-induced change in ionization, and to compensate for variations in instrument response from injection to injection, 17β-E2-d_3 was used as surrogate standards for 17α-E2 and 17β-E2. The Method Detection Limits (MDLs) of 17α-E2 and 17β-E2 for 2 L water samples were 0.05 ng L^{-1} and 0.08 ng L^{-1}, respectively.

3. Concentration Levels of 17α-E2 in the environmental matrices from various studies.

Table S2. Concentrations of 17α-E2 reported in different environment matrices.

Region	Concentration ($ng.L^{-1}$)	Matrices	Reference*
New Zealand	5 (max) ng. L^{-1}	Domestic STP effluent	Belfroid et al., 1999
	2.1 (max) ng. L^{-1}	Industrial STP effluent	Belfroid et al.,.1999
	3.0 (max) ng. L^{-1}	Surface water	Belfroid et al., 1999
USA	74 (max) ng. L^{-1}	139 Streams	Kolpin et al., 2002
Chester County, Pennsylvania. USA	0.04 (min) ng. L^{-1} 7 (max) ng. L^{-1}	21 Streams	Velicu and Suri, 2009
China	85.2 (med) ng. L^{-1}	STP inffluent	Zhou et al., 2009
	33.8 (med) ng. L^{-1}	STP effluent	Zhou et al., 2009
	0.02-0.91 ng. L^{-1}	Surface water	Chang et al., 2009
New Zealand	9.5 ng. L^{-1}	STP effluent	Sarmah et al., 2006
	1028 (max) ng. L^{-1}	Dairy farm	Sarmah et al., 2006
	10.9 ng. L^{-1}	Piggery farm	Sarmah et al., 2006
	172 ng. L^{-1}	Goat farm	Sarmah et al., 2006
New Zealand	19-1028 μg. kg^{-1}	Dairy oxidation pond	Sarmah et al., 2006
	172 μg. kg^{-1}	Goat effluent (slurry)	Sarmah et al., 2006
	11 μg. kg^{-1}	Piggery oxidation pond	Sarmah et al., 2006
Paris	1.5 (min) ng. L^{-1} 17 (max) ng. L^{-1}	STP inffluent	Miege et al., 2009
	7.4 (med) ng. L^{-1} 0.1 (min) ng. L^{-1}		
	3 (max) ng. L^{-1}	STP effluent	
	0.8 (med) ng. L^{-1}		
Japan	2000 ng. L^{-1}	Cattle wastewater inlet	Furuichi et al., 2006
	650-680 ng. L^{-1}	Rawswine wastewater	Furuichi et al., 2006
	660 ng. L^{-1}	UASB outlet	Furuichi et al., 2006
	24 ng. L^{-1}	Trickling filter effluent	Furuichi et al., 2006
USA	About 1000 ng. L^{-1}	lagoon samples	Hutcins et al., 2007
	139 ± 7 μg. kg^{-1}	Dairy(press cake solids)	Raman et al., 2001
	603 ± 358 μg. kg^{-1}	Dairy, dry-stack(semisolid)	Williams et al., 2002
USA	289 ± 207 μg. kg^{-1}	Dairy, dry-stack(solid)	Williams et al., 2002
	370 ± 59 μg. kg^{-1}	Holding ponds	Williams et al., 2002
	890 ± 120 μg. kg^{-1}	Piggery (farrowing pit)	Williams et al., 2002
	About 2400 ng. L^{-1}	Dairy waste water flow	Zheng et al., 2008
California	1416 ± 104 μg. kg^{-1}	Dairy fresh manure	Zheng et al., 2008
	172 ± 9 μg. kg^{-1}	Dairy piled manure	Zheng et al., 2008
Tokyo	0.33 (max) ng. g^{-1}	Sediment	Isobe et al., 2006
China	2.4-4.2 ng. g^{-1}	Mollusk	Liu et al., 2009

***Reference:**

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