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Short communication

The development of an optimized sample preparation for trace level detection of 17α-ethinylestradiol and estrone in whole fish tissue

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ABSTRACT

The purpose of this study was to develop an optimized method for the extraction and determination of 17α-ethinylestradiol (EE2) and estrone (E1) in whole fish tissues at ng/g levels. The optimized procedure for sample preparation includes extraction of tissue by accelerated solvent extraction (ASE-200), lipid removal by gel permeation chromatography (GPC), and a cleanup step by acetonitrile precipitation followed by a hexane wash. Analysis was performed by gas chromatography/mass spectrometry (GC/MS) in negative chemical ionization (NCI) mode after samples were derivatized with pentafluorobenzoyl chloride (PFBCl). The method was developed using high lipid content wild fish that were exposed to the tested analytes. The whole procedure recoveries ranged from 74.5 to 93.7% with relative standard deviation (RSD) of 2.3–6.2% for EE2 and 64.8 to 91.6% with RSD of 9.46–0.18% for E1. The method detection limits were 0.67 ng/g for EE2 and 0.68 ng/g for E1 dry weight. The method was applied to determine EE2 levels in male goldfish (*Carrasius auratus*) after a 72 h dietary exposure. All samples contained EE2 averaging 1.7 ng/g (±0.29 standard deviation, $n = 5$). This is the first optimized protocol for EE2 extraction from whole fish tissue at environmentally relevant concentrations. Due to high sensitivity and recovery, the developed method will improve our knowledge about the environmental fate and uptake of synthetic steroidal estrogens in fish populations.

1. Introduction

Considerable progress has been made in the development of sophisticated techniques to extract, clean-up, and analyze steroidal estrogens from various environmental matrices including surface water, ground water, sewage effluents, sewage sludge, soil, and sediment [1–4]. However, very few studies have focused on the analysis of these molecules in invertebrates [5] or fish [6–8]. Optimized sample preparation protocols for the extraction of steroidal estrogens from fish tissues with high recoveries and at environmentally relevant concentrations are not available.

Fish tissue can be a very complex matrix to deal with because of its richness in lipids and proteins. Finding an efficient extraction method that successfully maximizes the extraction of the target analytes and minimizes matrix effects is a challenge [1]. Sonication in methanol followed by solid phase extraction purification and enzymatic hydrolysis were the main steps used to analyze three steroidal estrogens (estradiol E2, E1, and EE2) in roach (*Rutilus rutilus*) gonads exposed to sewage effluents for 10 days [7] however, the GC/MS analysis that was performed in this study aimed only to confirm the identity of the active fractions detected by a bioassay. Länge et al. [8] measured EE2 concentrations by radioimmunoassay in whole body fathead minnow (*Pimephales promelas*) after a laboratory exposure. However, the main criticism for bioassays for estrogen analysis is low accuracy and limited sensitivity [9]. We have used ASE, GPC, and Florisil clean-up to prepare samples for analysis by LC/MS in a pilot study to determine EE2 for the first time in wild fish [6]. Although successful analysis was achieved, the major drawback for that method was low recoveries, which ranged from 25 to 30%.

Here we developed an optimized sample preparation and GC/MS procedure for the detection and quantification of EE2 and E1 in whole fish homogenates. The ASE was the preferred extraction technique due to its robustness, full automation, and lower solvent and time consumption [10]. Whole method recovery was the main criterion for the method validation. This study is the first to provide an optimized sample preparation protocol with high recovery and sensitivity for environmentally relevant measurements of EE2 and E1 in wild fish using ASE and GC/MS.

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Table 1
Accelerated solvent extraction conditions used during method development.

ASE parameter	Extraction conditions
Static	8 min
Cycles	1
Solvent	DCM
Heat	5 min
Temperature	70 °C
Pressure	2000 psi
Purge	120 s

2. Experimental

2.1. Standards and reagents

^{12}C and ^{13}C standards for E1 (99% purity) and EE2 (99% purity) were all purchased from Cambridge Isotope Lab., Inc. (Andover, MA, USA). Stock solutions (500 ng/mL) were prepared in nonane (Sigma–Aldrich, St. Louis, MO, USA.) and stored at 4 °C. Dichloromethane (DCM), methanol (MeOH), acetone and hexane, were Optima® grade (Fisher Scientific, Brockville, ON, Canada) while acetonitrile and water were Chromsolv grade (Fluka, Mississauga, ON, Canada). PFBCl, nonane, anhydrous Na_2SO_4, and potassium hydroxide (KOH) were all from Sigma–Aldrich (St. Louis, MO, USA). Hydromatrix was from Varian Inc. (Mississauga, ON, Canada).

2.2. Fish

Shorthead redhorse sucker (*Moxostoma macrolepidotum*) (ShRHSs) samples used in the spike recovery experiments were collected in June 2009 near the City of Montreal sewage treatment plant. Goldfish (*Carassius auratus*) were purchased from Aleong's International Inc. (Mississauga, Ontario, Canada). Fish were acclimatized to 18 ± 1 °C in 70-L holding tanks under a natural photoperiod and fed with standard floating trout pellets. The exposure experiment was conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee.

2.3. Goldfish feeding exposure

Male goldfish ($n = 5$) weighing 23.89 g ± (4.21 SD) were exposed to EE2 through food for validation purposes. EE2 was dissolved in ethanol and mixed with fish food to achieve 100 ng/g. The ethanol was thoroughly evaporated before the exposure. Fish were fed 1% body weight three meals a day for three days. Anesthetized fish with MS-222 (Sigma–Aldrich, St. Louis, MO, USA) were sacrificed by trans-spinal sectioning, put in falcon tubes (50 mL), and then placed on ice for 10 min to allow blood to coagulate. Gastrointestinal tracts were carefully removed. Samples were frozen at −20 °C and subsequently freeze-dried. Samples (2 g) were spiked with ^{13}C EE2 (50 ng) prior to the extraction in ASE cells (11 mL) as described below (Fig. 1).

2.4. Sample preparation

2.4.1. Spike recovery

All extractions were performed with a pressurized liquid extraction system (ASE-200, Dionex Corporation, Sunnyvale, CA, USA) and our optimum operation conditions are listed in Table 1. Two main experiments were conducted to assess the effect of the extraction cell size and hydromatrix on analytes' recovery. In the first experiment, freeze-dried tissue (2 g) was mixed with hydromatrix (6 g), spiked separately with ^{12}C E1 (50 ng/g) and ^{12}C EE2 (125 ng/g), and placed in the extraction cell (33 mL). In the second experiment, tissue (2 g) was placed in the extraction cells (11 mL) without hydromatrix and spiked with ^{12}C E1 at 1.25, 2.5, 5 ng/g and ^{12}C EE2 at 12.5, 25, 50 ng/g. Hydromatrix and Na_2SO_4 were used to produce the method blanks for quality control. DCM was the extraction solvent. The extracts (~20 mL) were filtered by polytetrafluoroethylene (PTFE) filters (0.45 μm) using disposable syringes (3 mL) (cat. # 1481727) (Henke Sass Wolf, Germany) and then evaporated by Turpo-Vap® II evaporation tubes (Zymark Hopkinson, MA, USA) to ~1 mL at 35 °C. Evaporation tubes were rinsed with DCM (2 mL) and pooled with the samples that were collected in preparative-LC vials (6 mL) and kept in the fridge at 4 °C.

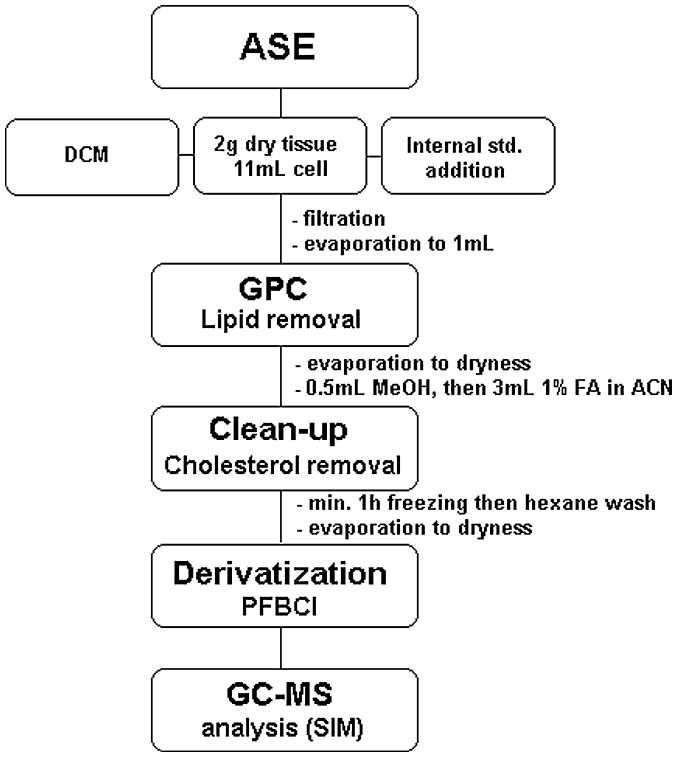

Fig. 1. A schematic for the optimized sample preparation protocol.

2.4.2. Gel-permeation chromatography

Samples were subjected to GPC following a slightly modified U.S. EPA method 3640A [11]. Briefly, samples were fractionated by two Envirogel™ GPC cleanup columns connected in tandem (19 mm × 150 mm → 19 mm × 300 mm) (Waters Corporation, MA, USA). DCM was the mobile phase delivered at a flow rate of 7 mL min^{-1} and a pressure of 57 ± 1 bar using an Agilent 1200 Series preparative HPLC system. Fractions were automatically collected at timed intervals (Fig. 2). Three to four injections were performed per sample and the injection volume was optimized based on the column loading limits of 10 mg/100 μL (lipid/solvent).

2.4.3. Sample clean-up and derivatization

Proton nuclear magnetic resonance (NMR) analysis was performed using a Bruker Avance 400 NMR spectrometer to elucidate the major matrix factor within the GPC cleaned fractions. The obtained NMR spectra were identified as cholesterol (Fig. 2) by a comparative approach with previously reported data [12]. Thus, all fractions for each sample were pooled in Turbo-Vap® tubes, evaporated to dryness, reconstituted in MeOH (0.5 mL), and transferred using Pasteur pipettes to disposable PYREX test tubes (16 mL) (cat. # 99445-16) (Corning Incorporated, NY, USA), which permitted removal of any precipitates. The Turbo-Vap® evaporation tubes were washed with 1% formic acid in acetonitrile (3 mL), combined with samples in the disposable test tubes, and then placed in a

Table 2
The overall sample preparation protocol performance and reproducibility of triplicate spiked ShRHS samples.

Packing material	Amount (g)	ASE cell size (mL)	Spiking level (ng/g)		Rec. (%)	SD	RSD (%)	Rec. (%)	SD	RSD (%)
			EE2	E1	EE2			E1		
Non-optimized										
T, H	2, 6	33	50	50	27.74	5.8	21	25.18	3.3	13.27
T, H	2, 6	33	250	250	43.02	9.2	21.3	54.50	11.1	20.3
Optimized										
T	2	11	12.5	1.25	74.53	1.72	2.31	64.8	6.13	9.46
T	2	11	25	2.5	81.45	2.62	3.21	57.01	8.7	15.26
T	2	11	50	5	93.70	5.8	6.18	91.64	0.17	0.18

Rec.: recovery (%), SD: standard deviation, RSD: relative standard deviation, T: tissue from ShRHS, and H: hydromatrix.

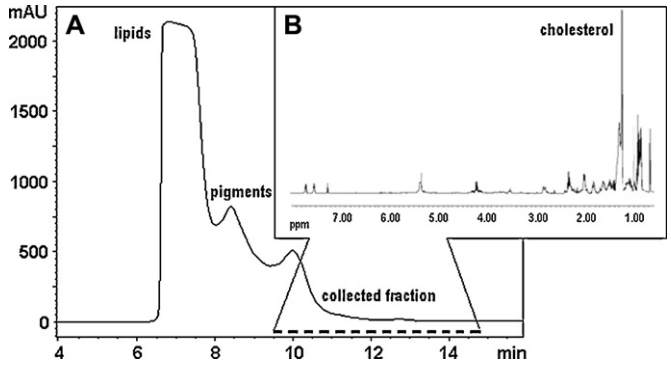

Fig. 2. A chromatogram showing lipid and cholesterol removal from whole fish extracts: (A) a preparative-HPLC spectrum of a 1 mL sample injection and (B) a proton NMR spectrum of the collected fraction showing cholesterol as a co-eluted matrix factor. The dashed line indicates the time based fraction collection window.

−20 °C freezer for at least 1 h or overnight to precipitate cholesterol out of the solution. After this step, samples were centrifuged for 15 min at 4000 rpm at 4 °C and then transferred to clean tubes. Cholesterol appeared as suspended white flakes during the freezing step. Hexane (2 mL) was used in a wash step to liquid–liquid extract any trace of the non-polar matrix. After a short gentle vortex, phase separation occurred and the upper hexane layer discarded, this procedure was performed twice. Samples were dried under a gentle stream of N_2 and kept at 4 °C. ^{13}C E1 and EE2 (50 ng of each) were added for recovery assessment prior to the derivatization for the GC analysis as previously described [13].

2.5. GC/MS analysis

The pentafluorobenzoyl-derivative of estrogen was separated and detected by GC/MS according to a method previously described [13] after a few modifications. Samples (4 μL) were injected in splitless mode onto an Agilent 6890 gas chromatograph with a Zebron ZB5-MS column (10 cm guard + 29.7 m × 250.00 μm × 0.25 μm), Phenomenex Inc. (Torrence, CA, USA). The injector was set at 280 °C and the oven temperatures were programmed as follows: 80 °C for 1.24 min, 24 °C min^{-1} until 200 °C and held for 0.5 min, 73 °C min^{-1} until 245 °C and held for 5 min, and then 1 °C min^{-1} to 260 °C. Helium (2 mL min^{-1}) was the carrier gas and methane (65 cm s^{-1}) was the reagent gas. The transfer line temperature was set at 330 °C. A Hewlett Packard 5973 mass spectrometer, with a quadrupole and ion-source temperatures set at 100 °C and 200 °C, respectively, was used for quantification of the analytes in NCI mode. The following quantification ions were used in selected ion monitoring (SIM) mode: *m/z* 490 (^{12}C EE2), *m/z* 492 (^{13}C EE2), *m/z* 464 (^{12}C E1), and *m/z* 466 (^{13}C E1). Chromatographic data were collected and analyzed using Agilent MSD Chemstation Data Analysis Software. All quantifications were based on an isotopic dilution method described by de Hoffmann and Stroobant [14].

3. Results and discussion

3.1. Spike recovery

As shown in Table 2, EE2 recovery was 27% ± (5.8 SD) while E1 recovery was 25.2% ± (3.3 SD) when extraction cells (33 mL) were packed with 2 g tissue with previously determined lipid content (ShRHSs, 21.7% dry weight) mixed with hydromatrix (6 g) and spiked with both estrogens at 50 ng/g. Recoveries increased at higher spiking concentrations (125 ng/g) of both analytes, EE2 recovery was 43% ± (9.2 SD) while E1 recovery was 54.5% ± (11.1 SD).

To further improve the overall method recovery of our target compounds, another experiment was conducted in which hydromatrix was eliminated and the smaller extraction cells (11 mL) were used instead. This approach significantly improved the recovery of both analytes. The recovery of EE2 was linearly correlated with the spike concentration (R^2 = 0.975): 74.53% ± (1.72 SD) for 12.5 ng/g, 81.45% ± (2.62 SD) for 25 ng/g, and 93.7% ± (5.8 SD) for 50 ng/g while E1 recoveries were 64.8% ± (6.13 SD) for 1.25 ng/g, 57% ± (8.7 SD) for 2.5 ng/g, and 91.64% ± (0.17 SD) for 5 ng/g. The RSD values for EE2 below 6.2% for EE2 and 15.3% for E1 indicating good reproducibility.

Improved recoveries were achieved after the optimization of some critical steps during sample preparation. Firstly, freeze drying the whole fish homogenates was appropriate in light of the low vapor pressure of steroidal estrogens (3×10^{-8} to 9×10^{-13} Pa) [15] and to avoid multiple freeze–thaws during sample preparation, which should reduce enzymatic activity that might affect the stability of these analytes. Freeze-drying was associated with high steroidal estrogen recoveries in sediment samples [16–18]. Secondly, the elimination of hydromatrix resulted in higher recoveries suggesting that it binds with our analytes. Thirdly, the NMR identification of cholesterol as the major matrix factor after the GPC fractionation enabled us to include a precipitation step with acetonitrile. The significance of this step is even more apparent when the sample is analyzed by liquid chromatography because the polar mobile phase may precipitate cholesterol in the separation column over time and reduce its lifespan. Lastly, the hexane wash was also a critical step to help remove any non-polar matrix residuals that could form emulsions. Qualitative full scan analysis showed that after the derivatization step, the samples were fairly clean, however the samples may require additional clean-up if liquid chromatography is chosen for analysis.

3.2. Validation of the sample preparation method

Prior to the analysis of either the spike-recovery samples or the laboratory EE2 exposed goldfish, the GC/MS linearity and limits of detection were assessed by injection of standards. Within the tested range (0.004–1.0 ng, on column) for EE2 and (0.0008–0.2 ng, on column) for E1 the linearity was excellent, with R^2 values of 0.999 for

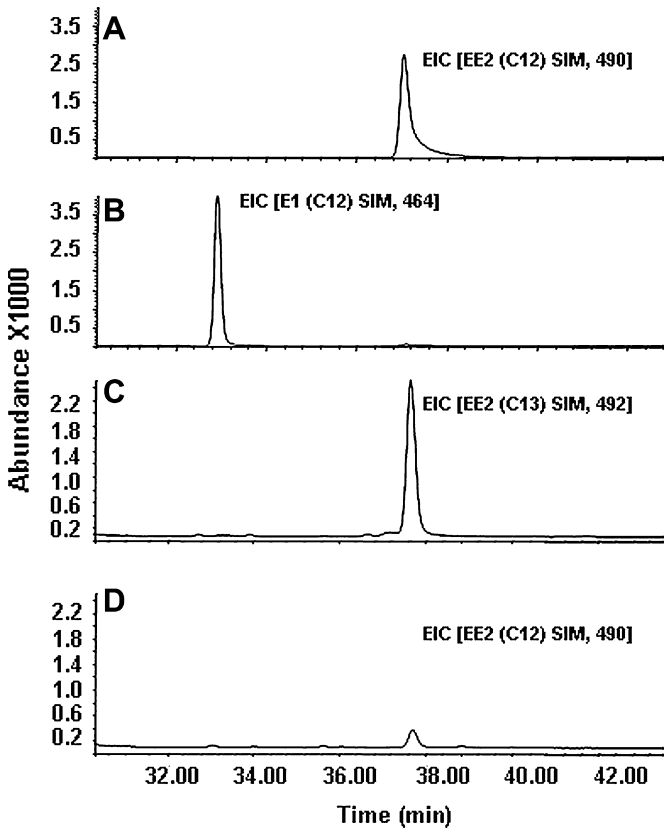

Fig. 3. Extracted ion chromatogram (EIC) showing the response of (A) EE2 standard injected at 1 ng on column and (B) E1 standard injected at 0.2 ng on column. (C and D) A sample of 2 g goldfish extracts showing the internal EE2 ^{13}C spiked standard spiked at 50 ng and the quantified endogenous EE2 in the sample, respectively.

both analytes. The intra-day RSD values ranged from 4.3 to 7.5% for EE2 and 4.3 to 11.7% for E1 for 4 consecutive injections. The instrument detection limits (LODs, 3:1 signal:noise) were 4 and 0.8 pg (on column) whereas the quantification limits (LOQs, 10:1 signal:noise) were 12 and 3.2 pg (on column) for EE2 and E1, respectively. The method detection limits (MDLs) were 0.67 ng/g for EE2 and 0.68 ng/g for E1, respectively.

The commercial fish diet that was spiked with EE2 contained 108 ng/g ± (3.4 SD) which was very close to the target concentration of 100 ng/g. The results demonstrated that EE2 can be extracted and quantified not only from spiked wild-fish samples but also from laboratory exposed goldfish tissue (Fig. 3). Our optimized protocol showed good accuracy, selectivity, and sensitivity. Each exposed fish ($n = 5$) accumulated EE2 to measurable levels whereas two control samples that were not exposed by any means did not show any EE2 signal. The maximum determined EE2 concentration was 2.2 ng/g whereas the minimum concentration was 1.53 ng/g with the mean of 1.7 ng/g ± 0.29 (SD) (dry weight).

4. Conclusions

A sample preparation method consisting of ASE, GPC fractionation, sample purification, and GC/MS analysis is described. The proposed method enables the determination of estrogen levels in whole fish tissues at very low and environmentally relevant ng/g dry tissue concentrations. To date, this method is the first optimized protocol for the determination of EE2 in whole fish tissue with high recovery and low method detection limits. This method is also expected to work with other types of samples like fish organs or invertebrates exposed to EE2 without considerable modifications. This method will help to further expand our knowledge about the uptake and elimination of EE2 in fish from natural environments. The method has been successfully applied to measure EE2 levels in laboratory exposed goldfish where it was detected at an average concentration of 1.7 ng/g ± (0.29 SD).

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