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# Investigation of bioaccumulation profile of oestrogens in zebrafish liver by hollow fibre protected liquid phase microextraction with gas chromatography–mass spectrometric detection

Sivarajan Kanimozhi[a], Chanbasha Basheer[b], Shanmugam Neveliappan[c], Kelvin Ang[a], Feng Xue[a], Hian Kee Lee[a,*]

[a] Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore
[b] Department of Chemistry and Center for Nanotechnology, King Fahd University of Petroleum and Minerals, KFUPM Box 1509, Dhahran 31261, Saudi Arabia
[c] School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, 535 Clementi Road, Singapore 599489, Singapore

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ABSTRACT

The applicability of hollow fibre protected liquid phase microextraction (HF-LPME) for the determination of three oestrogens, namely estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) from individual zebrafish liver samples, in a bioaccumulation study on these organisms, is reported. The oestrogens were extracted from single, mechanically crushed and minced livers from fish that were heaved in tubes containing water spiked at low concentration of the analytes. Extraction was performed with ~3 μL of toluene contained in the hollow fibre. In order to achieve high extraction efficiency, the parameters that could affect the effectiveness of HF-LPME were optimized, i.e. the extracting organic solvent, extraction time, stirring speed and pH of the aqueous phase. For gas chromatography/mass spectrometry (GC/MS) analysis, injection port derivatization of the oestrogens with bis(trimethylsilyl)trifluoroacetamide was conducted. Under the most favourable extraction and derivatization conditions, enrichment factors of 158–279 were obtained. Linearity of the HF-LPME–GC/MS method was evaluated from 1 to 50 μg/L and the coefficient of determination ($r^2$) ranged from 0.9687 to 0.9926. The LODs were between 0.017 and 0.033 μg/L (at a signal to noise ratio of 3) with relative standard deviations (RSDs, analytes spiked at 5 μg/L) of between 15 and 17% ($n = 3$).



## 1. Introduction

The release of endocrine disrupting compounds into the aquatic environment has been a serious threat to organisms [1,2]. These compounds interfere with the endocrine system of the aquatic organisms and cause undesirable physiological changes to them. One class of these endocrine disrupting compounds consist of the hormone oestrogens such as estrone (E1), 17β-estradiol (EE2), estriol (E3), diethylstilbestrol (DES) and 17α-ethinylestradiol (EE2). EE2 is a synthetic oestrogen used frequently in pharmaceutical products such as birth control pills as well as in hormone replacement therapy for women [3,4]. Thus, women who are on such medication may excrete this synthetic oestrogen together with the other naturally occurring oestrogens. These excreted oestrogens may accumulate in aquatic organisms living in such polluted environments and these can extend devastating effects on these organisms [5–7].

Oestrogens are female sex hormones whose structures bear the polycyclic steroid structure and are known to cause abnormalities in reproduction of wildlife, particularly feminization of male fishes [8]. In the case of zebrafish (*Danio rerio*), the protein expression of male zebrafish is altered due to the presence of oestrogens [9]. Zebrafish are increasingly being used to study the effects of chemicals and pharmaceuticals in the environment [10,11]. Male and female zebrafish are sensitive to low concentrations of waterborne oestrogens. Since the zebrafish genome resembles that of human the prediction of rate of accumulation of oestrogens in zebrafish can reveal the effects of xenobiotic oestrogens on humans [12]. There is a direct correlation between the rate of bioaccumulation of oestrogen in zebrafish and the extent of xenobiotics oestrogenic effects such as vitellogenin (VTG) induction and physiological changes in this species [13]. Earlier studies on fishes exposed to xenooestrogens mainly dealt with the quantification of VTG concentration and its effects on fish. Hence there is a need for a rapid analytical technique to estimate the bioaccumulation and hence its subsequent effects on fish that has undergone oestrogenic exposure. The objective of the current study was to establish a sensitive microextraction technique to quantify

* Corresponding author. Tel.: +65 6516 2995; fax: +65 6779 1691.
*E-mail address:* chmleehk@nus.edu.sg (H.K. Lee).

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the bioaccumulated oestrogens with low exposure concentration. This estimation of accumulated oestrogens along with a further study on induced VTG and its effects will provide a clear depiction of xenoestrogenic effects on fishes. In this regard, we intended to develop a simple analytical method to determine the bioaccumulation of oestrogens in zebrafish liver. Oestrogens are present in micrograms to sub-micrograms per litre levels in the aquatic environment [6,14] and the bioaccumulation factor in the fish could be much less; therefore, an effective and fast extraction method is needed for the oestrogens.

Traditional methods of extraction such as liquid–liquid extraction (LLE) and solid phase extraction (SPE) uses moderate to large volume of organic solvents, after which the extracts have to undergo further pre-concentration prior to analysis. In recent years, liquid phase microextraction (LPME) has been used for the extraction of a variety of organic compounds such as alkylphenols [15] phthalate esters [16] and pesticides [17]. Various modes of LPME have been developed such as single drop microextraction (SDME) [18–21]. Headspace SDME [22,23] has been used mainly for the more volatile organic compounds. Although high enrichment factors can be achieved using SDME, the single drop of organic solvent is potentially unstable and may be easily dislodged from the syringe needle during the extraction process. Polymer coated hollow fibre LPME [24], another variant of LPME, makes use of the affinity of certain analytes to some polymers used as sorbents. Another LPME mode, hollow fibre protected (HF-LPME) represents a simultaneous extraction, cleanup and pre-concentration approach [25–28]. The organic solvent used for extraction is protected by the hollow fibre. In this work, for the first time, HF-LPME has been developed for single zebrafish liver. Naturally occurring oestrogens, estrone (E1) and 17β-estradiol (E2), and the synthetic oestrogen 17α-ethinylestradiol (EE2) bioaccumulation on zebrafish were investigated. Oestrogens, being steroidal compounds, are non-volatile and thus not suitable for direct gas chromatography/mass spectrometry (GC/MS) analysis. Hence, derivatization of the oestrogens, after extraction with HF-LPME, was needed. The conventional offline derivatization was not a feasible choice as the organic extract volume using HF-LPME was very small (3 μL). Therefore for the first time with oestrogens, we made an attempt to carryout derivatization conducted in the injection port of the GC/MS system after extraction.

## 2. Experimental

### 2.1. Chemicals and materials

HPLC-grade methanol, *n*-hexane, dichloromethane were purchased from Tedia Company (Fairfield, OH, USA) and ethyl acetate was from Riedel-DeHaen AG (Seelze-Hannover, Germany). Toluene was purchased from Fisher Scientific (Loughborough, UK). Derivatization reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA), hydrochloric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). The high purity standards (99%) of estrone, 17β-estradiol and 17α-ethinylestradiol were from Sigma Chemical (St Louis, MO, USA). Ultrapure water was prepared on a Nanopure water purification system (Barnstead, Dubuque, IA, USA).

Q3/2 Accurel polypropylene hollow fibre membrane with an inner diameter of 600 μm, wall thickness of 200 μm and wall pore size of 0.2 μm was purchased from Membrana (Wuppertal, Germany). A 10 μL GC syringe, with cone-shaped needle tip, from SGE (Sydney, Australia) was used for manual sample injection into the GC/MS system. Stock standard solutions were prepared in methanol at 1000 mg/L of each analyte and working standards were prepared by spiking appropriate volume of the stock solution with methanol.

### 2.2. Hollow fibre–liquid phase microextraction

Hollow fibres were cut into 1.2 cm segments for HF-LPME. The approximate internal volume of such a 1.2 cm segment was 3 μL. The 10 μL GC syringe was rinsed with methanol and the organic solvent used for extraction before being set up for the next extraction process. Approximately 3 μL of organic solvent was withdrawn into the GC syringe and the needle of the syringe was inserted into hollow fibre to about 0.2 cm of the tip of the syringe needle was covered by it. The hollow fibre was then dipped into the organic solvent for 10 s to impregnate its pores. The entire hollow fibre–syringe assembly was clamped on a retort stand, with the hollow fibre immersed in 1.5 mL of the aqueous sample phase contained in a 2 mL vial. The organic solvent in the GC syringe was released into the channel of the hollow fibre after which the stir bar in the aqueous phase was activated and at the same time, the stopwatch was started to measure extraction time. After extraction, the stirring was stopped, and the organic solvent, was retracted into the GC syringe. The hollow fibre was removed, and the organic extract in the syringe was adjusted to 2 μL (the rest was discarded). Using the same syringe, 2 μL of BSTFA was withdrawn, making the total volume in the GC syringe 4 μL. The entire 4 μL was injected into the GC–MS for injection port derivatiztion and analysis.

### 2.3. Experimental animals

Zebrafish were heaved at Ngee Ann Polytechnic, School of Life Sciences & Chemical Technology, as part of the project collaboration. Approximately four hundred adult zebrafish were received from the local market and male zebrafish were separated from female fish by visual observation. Male zebrafish were maintained in 40 L glass aquaria with 40 fishes per tank. Each aquarium was individually heated using a 100 W aquarium heater to maintain a temperature of 26–29.8 °C. Fish were kept in filtered tap water, which was purified with activated charcoal and aerated. Aeration and filtration were provided using sponge filters. Tanks were fed with a flow through system that provided 1 L/h of carbon filtered, and dechlorinated water. Fish were fed a diet of Aquatox flake food (Aquatic Ecosystems, Apopka, FL, USA) in the morning and in the evening. Fish were maintained on a photoperiod of 16 h light:8 h dark. The pH ranged from 7.0 to 7.6 throughout the duration of the experiment, and ammonia concentrations were non-detectable. Fish were allowed to acclimate to laboratory conditions for 4 weeks prior to experiments. Each tank was spiked at three different concentrations (0.1 μg/L, 1 μg/L of 50 μg/L) of oestrogens (three replicate tanks for each concentration) and fresh oestrogen standards were added at the time of water change.

The fishes were sampled every week (three samples from each tank) and were sacrificed in the Polytechnic's Life Sciences Laboratory, and their livers were removed. The livers were transported to NUS in an ice box. A single liver was used for extraction. The wet weight of each liver used ranged from 15 to 20 mg. The liver was crushed and minced using a metal spatula, followed by the addition of 1.5 mL of hydrochloric acid solution (pH 2) and the solution was sonicated for 20 min. The mixture was stirred for 10 min and after which it, as a slurry, was directly extracted by HF-LPME as described above.

### 2.4. GC/MS analysis

Analysis was carried out using a Shimadzu (Kyoto, Japan) QP2010 GC–MS system equipped with a DB-5MS fused silica capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm, from J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 2.1 mL/min. Two microliters of extract together with 2 μL BSTFA were injected into the splitless injection port under

splitless mode and a sampling time of 2 min was allowed to take place (i.e. sample and derivatization agent were retained in the injection port for 2 min). Since the injection port is heated to high temperature (300 °C) and pressurized, the holding time of 2 min would allow the oestrogens and BSTFA to react. The MS interface temperature was set at 280 °C. The GC temperature program was as follows: initial temperature of 90 °C (held for 2 min); 10 °C/min to 220 °C (held for 8 min); increased at 10 °C/min to 300 °C (held for 2 min). A standard solution of 1 μg/L each analyte was initially analyzed in scan mode (at *m*/*z* range between 50 and 500) to identify the retention times and peak resolutions. From the scan mode chromatograms and mass spectrum, the most abundant *m*/*z* ion for each analyte was selected as the quantification ion (E1; 342 $[M+72]^+$, E2; 416 $[M+72+72]^+$, EE2; 368 $[M+72]^+$) and the next 2 abundant ions were selected as confirmatory ions. Subsequent GC–MS analysis was done using selected ion monitoring (SIM) mode.

## 3. Results and discussion

### 3.1. Injection port derivatization

BSTFA replaces the proton of the OH group with the trimethylsilyl (TMS) group, making these compounds volatile by reducing the occurrence of hydrogen bonding between molecules, thus allowing GC–MS analysis. Estrone with only the phenolic OH group gives the TMS-E1 derivative. 17β-estradiol has two OH groups, one phenolic OH and the other alcoholic OH; both of these OH groups react with BSTFA to give the di-TMS-E2 derivative [29]. 17α-ethinylestradiol also has both phenolic and alcoholic OH groups; however, the alcoholic OH does not react with BSTFA, probably due to the low nucleophilicity of the tertiary alcohol, and thus to a lesser extent it forms the TMS-EE2 derivative [30]. HF-LPME is an equilibrium extraction procedure. Therefore, we attempted to optimize the derivatization conditions with various combinations of extract volumes versus BSTFA volumes (see Supporting material, Figs. S1 and S2). Two microliters of extract solvent with 2 μL of BSTFA gave the largest peak area response. With injection port derivatization, we observed good peak shapes and complete derivatization of analytes (see Supporting material, Fig. S3). The use of BSTFA should be minimized as it causes bleeding of the column stationary phase by reacting with the polysiloxane on the liquid stationary phase. Hence, no more than 3 μL of BSTFA was used to preserve the GC column lifespan.

### 3.2. Extraction method optimization

To determine the most favourable HF-LPME conditions, ultrapure water spiked with 10 μg/L of each analyte was used. An univariant optimization approach was applied to achieve most satisfactory results.

#### 3.2.1. Solvent

The selection of organic solvent as extractant is crucial since different compounds have different partition coefficients in different solvents and such differences in solubility arise based on the nature of the compounds. The solvent would have to match the polarity of the compound as closely as possible so as to maximize extraction efficiency. The solvent should possess the following properties [27]. Firstly, it must be of low volatility to prevent solvent loss during the extraction process, compatibility with extraction analytes and secondly, the solvent should not be miscible with water [19,26]. In our method development, several common extraction solvents were tested and the solvent which gives the largest peak area in the chromatogram was considered the most favourable. Fig. 1 shown that

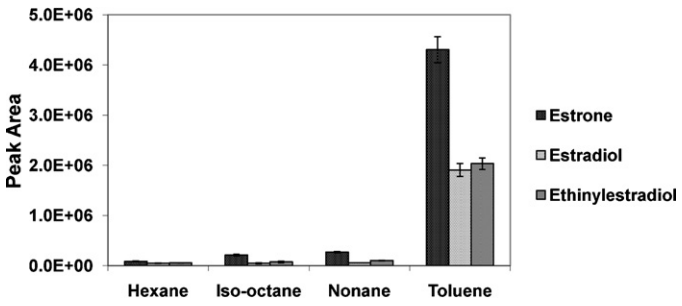


**Fig. 1.** Effect of organic solvents on HF-LPME. Extraction time 20 min, stirring speed at 500 rpm and 2 μL of BSTFA was used for injection-port derivatization. Sample pH and ionic strength were not adjusted.

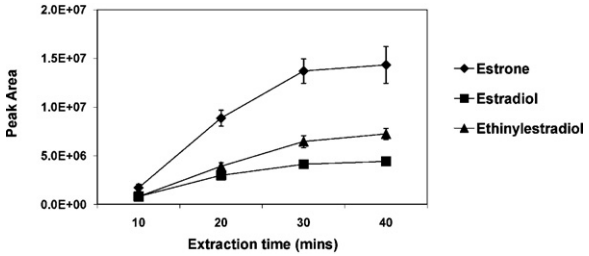


**Fig. 2.** Effect of extraction time on HF-LPME. Extraction solvent was toluene, stirring speed of 500 rpm and 2 μL of BSTFA was used for injection-port derivatization. Sample pH and ionic strength were not adjusted.

toluene was the most suitable extraction solvent when compared to hexane, iso-octane and nonane.

#### 3.2.2. Extraction time

Extraction of analytes from the aqueous sample phase into the organic phase in LPME requires equilibration between the two phases. The extraction process depends on diffusion which implies that the control of extraction time is crucial so as to achieve maximum extraction efficiency. Fig. 2 shows that a 30 min extraction time was sufficient as longer extraction time did not significantly yield larger peak areas, indicating that equilibrium was attained at this time.

#### 3.2.3. Stirring speed

During extraction, agitation is necessary to ensure that the maximum amount of analytes were being extracted into the organic solvent. The analyte molecules partition into the organic solvent enclosed within the hollow fibre via the pores of the fibre since they are hydrophobic in nature. Agitation constantly refreshes the surface layer of analytes around the hollow fibre, and thus analyte molecules are continuously being brought closer to the surface of the hollow fibre. Fig. 3 shows that stirring speed of 700 rpm yielded

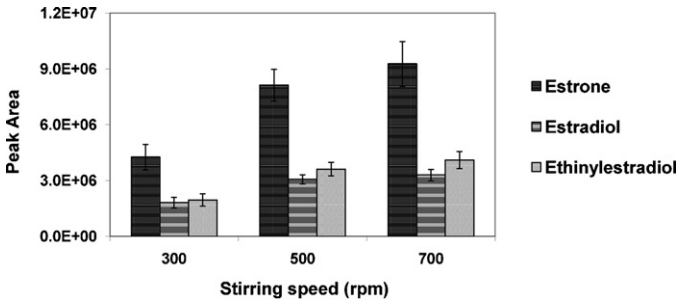


**Fig. 3.** Effect of stirring speed on HF-LPME. Extraction solvent was toluene, extraction time was 30 min and 2 μL of BSTFA was used for injection-port derivatization. Sample pH and ionic strength were not adjusted.

**Table 1**
Quantitative performance of HF-LPME on spiked water samples.

| Analyte | Linearity (μg/L) | % RSD ($n$ = 3) | LOD (μg/L) | LOQ (μg/L) | Coefficient of determination ($r^2$) | Enrichment factor (-fold) |
|---|---|---|---|---|---|---|
| E1 | 1–50 | 11.1 | 0.014 | 0.042 | 0.988 | 279 |
| E2 | 1–50 | 12.9 | 0.016 | 0.0472 | 0.973 | 158 |
| EE2 | 1–50 | 12.4 | 0.022 | 0.065 | 0.966 | 240 |

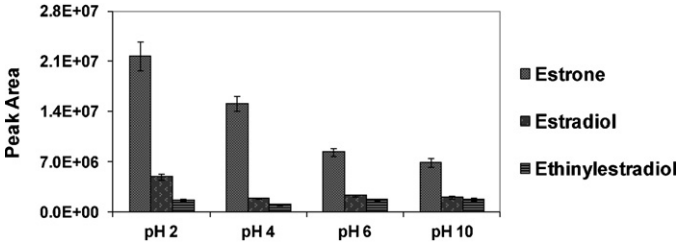


**Fig. 4.** Effect of pH on HF-LPME. Extraction solvent was toluene, extraction time was 30 min, stirring speed of 700 rpm and 2 μL of BSTFA was used for injection-port derivatization.

the largest peak area. At higher agitation speeds (e.g. 1000 rpm) approximately half of the organic solvent in the hollow fibre was lost from the hollow fibre, due to the vortex causing disturbance of the hollow fibre. Thus 700 rpm was selected as most favourable agitation speed.

### 3.2.4. Sample pH

The presence of acidic or basic functional groups on the analytes can affect extraction efficiency. Hence, the pH of the aqueous phase had to be adjusted so that the analytes remained in the non-ionized form, and thus favouring their partitioning into the organic phase. Fig. 4 shows that aqueous phase bearing pH of 2 yielded the largest peak area. The 3 oestrogens contain acidic phenolic protons, and under acidic conditions, remain unionized. This is indicated by their p$K_a$ values of between 10.34 and 10.46 indicating they are slightly acidic in nature. The non-ionized forms of the oestrogens (when pH value is 2) would be more hydrophobic than the ionized forms, thus, favouring their extraction by the organic solvent. Fig. 5 shows a typical GC–MS total ion chromatogram after extraction using optimized HF-LPME followed by injection port derivatization. We did not attempt to identify the peaks appearing after 28 min since they did not interfere with the analysis of the oestrogen whose peaks were sharp and symmetrical.

### 3.3. Extraction method evaluation

In order to access the practicality and suitability of this proposed HF-LPME method, the optimized extraction conditions were used to determine repeatability, linearity, limits of detection (LOD), limits of quantification (LOQ), enrichment factor and relative recovery. Spiked water samples were used. Repeatability was evaluated by triplicate analysis at the various analyte concentrations within the linear range of the extraction method. Satisfactory repeatability of relative standard deviations (RSDs) between 11.1 and 12.9% was obtained. The linearity of this extraction method was evaluated at five different concentrations, ranging from 1 to 50 μg/L (3 data points). The LODs were determined based on the signal to noise (S/N) ratio of 3, while the LOQs were calculated based on S/N = 10. The enrichment factors were calculated by comparing the peak areas of the respective analytes after extraction of spiked ultrapure water, and those of the analytes at the same concentration in the spiked sample which did not undergo the extraction process. All these analytical data are summarized in Table 1.

### 3.4. Real sample analysis

The optimized HF-LPME method was applied to zebrafish liver extraction. To assess the matrix effect of the method, uncontaminated blank zebrafish liver was spiked at 1 μg/L of oestrogens and HF-LPME recoveries were calculated. The extraction recoveries (i.e., E1, 84%; E2, 68% and EE2, 65%) indicated that there could be matrix effects. Lower extraction recoveries in real samples are due to matrix effect. At longer extraction time, most of the proteins and hydrophobic particles might blog the pores of the hollow fibre membrane and decreases the analyte transfer from sample to acceptor phase. Thus, separate calibration graphs were constructed based on uncontaminated zebrafish liver sample spiked between 1 μg/L and 50 μg/L of oestrogens. Table 2 shows the quantitative parameters of uncontaminated zebrafish spiked liver extract. For the bioaccumulation study, adult zebrafish were exposed to 0.1, 1 and 50 μg/L spiked water sample in the tank experiments. The fishes from each concentration tank were sacrificed every week and the liver tissue was removed and analyzed. Table 3 shows the

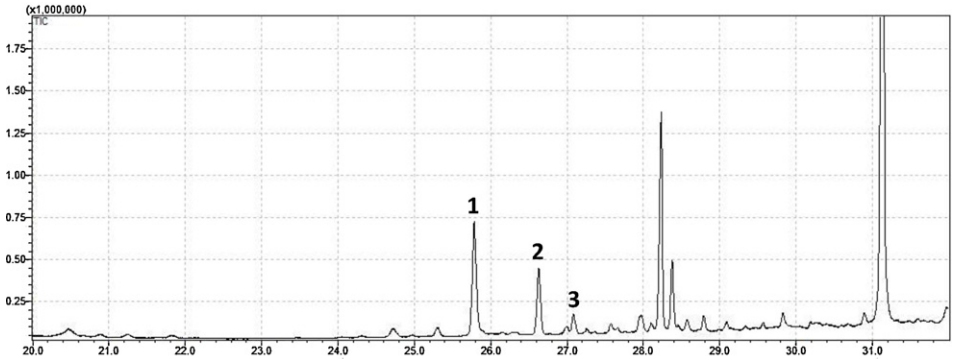


**Fig. 5.** shows a typical GC–MS total ion chromatogram after extraction of analytes extracted from real sample (single zebrafish liver) spiked with 5 μg/L of analytes using optimized HF-LPME followed by injection port derivatization. Peak identification: (1) TMS-E1, (2) di-TMS-E2, (3) TMS-EE2.

**Table 2**
Analytical performance of HF-LPME on single zebra fish extraction.

| Analyte | Linearity (μg/L) | Coefficient of determination ($r^2$) | Formula | % RSD ($n$ = 3) | LOD (μg/kg) | LOQ (μg/kg) |
|---|---|---|---|---|---|---|
| E1 | 1–50 | 0.9926 | $y = 320{,}307x - 82{,}729$ | 15 | 0.017 | 0.050 |
| E2 | 1–50 | 0.9831 | $y = 170{,}565x + 44{,}019$ | 17 | 0.023 | 0.070 |
| EE2 | 1–50 | 0.9687 | $y = 79{,}990x - 27{,}414$ | 18 | 0.033 | 0.100 |

**Table 3**
Bioaccumulation profile of oestrogen on individual zebra fish exposed in tank experiments at different interval of time ($n$ = 3).

| Week | Exposure concentration[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 50 μg/L | | | 1 μg/L | | | 0.1 μg/L | | |
| | E1 | E2 | EE2 | E1 | E2 | EE2 | E1 | E2 | EE2 |
| *Detected amount in single liver (μg/kg)* | | | | | | | | | |
| 1 | 0.12 | ND | ND | ND | ND | ND | ND | ND | ND |
| 2 | 0.11 | ND | ND | ND | ND | ND | ND | ND | ND |
| 3 | 0.12 | 0.23 | ND | ND | ND | ND | ND | ND | ND |
| 4 | 0.2 | 0.29 | 0.24 | 0.24 | ND | ND | ND | ND | ND |
| 5 | 0.29 | 0.33 | 0.25 | 0.3 | ND | 0.37 | ND | ND | ND |
| 6 | 0.4 | 0.41 | 0.13 | 0.3 | 0.14 | 0.33 | ND | ND | ND |
| 7 | 0.48 | 0.54 | 0.38 | 0.38 | 0.33 | 0.4 | ND | ND | ND |
| 8 | 0.5 | 0.51 | 0.4 | ND | 0.31 | 0.44 | 0.11 | ND | ND |
| 9 | 0.52 | 0.58 | 0.31 | 0.33 | 0.46 | 0.42 | 0.09 | ND | ND |
| 10 | 0.54 | 0.65 | 0.32 | 0.37 | 0.51 | 0.45 | ND | 0.11 | ND |

ND – not detected.
[a] Deviation > 18%.

oestrogen accumulation by the treated zebrafish liver over a 10-week period. The results show that there was a gradual increase in the accumulated oestrogen concentration over time. The oestrogens were not detected from the liver tissues of low concentration (0.1 μg/L) tank in the early weeks. It seems at low exposure concentration the fishes did not accumulate detectable levels of oestrogen. Moreover, reduction of exposure concentration is a problem when working with lipophilic compounds such as EE2 since they can be adsorbed on the aquarium walls. It is believed that more studies are required to improve the detection limits of the method to quantitate even lower concentrations of oestrogens. It is clear, however, that HF-LPME with GC–MS is a feasible method to determine oestrogen from individual zebrafish. Composite samples are not necessary.

## 4. Conclusion

In this study, for the first time, hollow fibre protected liquid phase microextraction (HF-LPME) was effectively applied to obtain the bioaccumulation pattern of oestrogen on zebrafish liver by conducting tank experiments. The extraction technique was optimized for the extraction of three oestrogens from a single zebrafish liver samples followed by injection port derivatization and GC–MS analysis. The HF-LPME method was simple to use, and allowed good enrichment factors by extracting analytes from 2 mL slurry sample into 3 μL of organic solvent. Good linearity with low limits of detection in the range of 0.017–0.033 μg/L and satisfactory repeatability with RSD of 15–18% were exhibited for spiked zebrafish liver samples. The results show that the technique is capable of determining accumulated low concentration oestrogens from single zebrafish liver and is feasible approach to determining the contaminant bioaccumulation profile of these organisms. Further microextraction studies will be conducted in our laboratory to improve the method sensitivity, and investigate the effect of bioaccumulation of these compounds and their correlation to physiological changes by investigating vitellogenin levels in these organisms.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.10.008.

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