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Improved method for analyzing the degradation of estrogens in water by solid-phase extraction coupled with ultra performance liquid chromatography-ultraviolet detection

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Abstract

We established an improved method for the determination of four estrogens including estriol (E3), 17β-estradiol (E2), 17α-ethynylestrodiol (EE2) and estrone (E1) in water. The method consisted of solid-phase extraction (0.5 L water) and subsequent analysis of analytes by ultra-performance liquid chromatography (UPLC) with an ultraviolet detector (UVD). Base-line separation was achieved for all studied estrogens using a column (50 mm × 2.1 mm) packed with 1.7 μm particle size stationary phase. Recovery was higher than 88% and detection limits ranged between 12.5–23.7 ng/L for the four estrogens, with the RSD ranging from 7% to 11%. The method was successfully applied to determine E2 and EE2 in simulated natural water, which found that about 70% of E2 was degraded (with a half-life of about 30 hr) within 48 hr and about 55% of EE2 was degraded (with a half-life of about 36 hr). Low levels of E1 were found, however E3 was undetectable during the process.

Key words: estrogens; base-lined separation; half-life; degradation

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Introduction

Large amounts of natural and synthetic reproductive hormones are released into surface waters, which cause adverse biological effects on fish and other wildlife. Estrogenic pollution is worse in developing countries due to ineffective wastewater treatment. The presence of natural and synthetic estrogens in surface water (Kolpin et al., 2002; López de Alda and Barceló, 2000) has attracted great interest from both professional researchers and the concerned public. Exposure to environmental estrogens has been shown to decrease sperm counts, increase rates of testicular, prostate, and breast cancers, and increase reproductive disorders in human males (Liu et al., 2004). A recent study demonstrated that estrogens at levels of 5–6 ng/L killed the entire fish population in a whole lake experiment in northwestern Ontario within three years due to the feminization of male fish (Pelly et al., 2003). Vitellogenin inducted by estrogenic compounds in aquatic environments has also been reported (Korsgaard and Pedersen, 1998; Gillespie and Peyster, 2004; Christensen et al., 1999; Verslycke et al., 2002; Patyna et al., 1999; Lv et al., 2006).

Sewage treatment plant (STP) effluent outfalls and animal waste or biosolids applied to agricultural fields can flow into nearby water bodies and infiltrate ground water (Maria and Barceló, 2001). Cattle and poultry manure had been reported as a source of 17β-estrodiol loadings in the environment (Kolpin et al., 2002), with 17α-ethynylestrodiol (EE2) also synthesized from 17β-estrodiol to form a stable oral contraceptive compound (Turan, 1996).

Generally, popular methods for analyzing estrogens include gas chromatography (GC) (Zuo et al., 2007; Zhang and Zuo, 2005) or high performance liquid chromatography (HPLC) (Wang et al., 2008). But complex derivative procedures have to be applied when analyzing compounds by GC, which can decrease recovery of the experiment and consume longer operation time. Over the last several decades, HPLC separation efficiency has progressed through improvement in silica-based particle manufacturing. In its scope of application, HPLC is dynamic from the capillary to preparative scale and versatile in detection techniques such as ultraviolet detector, fluorescence detector, electron capture detector and mass spectrometry, which provides analytical capabilities over a broad spectrum of compounds. Despite the impressive capabilities

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of modern HPLC systems, however, there are increasing requirements for higher throughput, sensitivity, and better chromatographic resolution.

Ultra-performance liquid chromatography (UPLC) is a new prospective development in liquid chromatography, and possibly able to meet the above requirements. From the van Deemter equations, we know that decreased particle size increases the separation efficiency of UPLC (Neue and Mazzeo, 2002; Wilson et al., 2005)

With UPLC™ technology and particle sizes of 1.7 μm, the HETP (Height equivalent to the theoretical plate) can be maintained, even at higher linear velocities. The UPLC takes full advantage of the flat section of the van Deemter plot for sub 2 μm stationary phase to generate higher chromatographic performance. When a 1.7-μm particle size was used, sensitivity increased 3–5 folds compared to conventional HPLC with a 5-μm particle size.

Theoretically, 17β-estradio (E2) and EE2 are hydrophobic organic compounds, which are unstable in the environment with low volatility (Ying et al., 2002). In natural water, E2 can be oxidized to estone (E1) by microorganisms at 20°C with half-lives of 0.2–9 days, and E1 can be further degraded at a similar rate (Jürgens et al., 2002). Compared to E2, EE2 is much more resistant to biodegradation in natural water (Jürgens et al., 2002).

While research has been focused on the degradation of estrogens, including the photochemical and microbial degradation (Coleman et al., 2005; Feng et al., 2005), no toxicology studies have been conducted on the exact concentration of estrogens during the exposure cycle. Therefore, a fast, high throughput, and highly sensitive method was established to separate and detect four estrogens by SPE-UPLC-UV. It was applied to examine E2 and EE2 changes in simulated natural water to determine the kinetic processes and decomposition products.

1 Materials and methods

1.1 Chemicals

Steroids were purchased from Sigma/Aldrich (USA). Individual stock solutions of estriol (E3), E2, EE2, and E1 were prepared by dissolving an appropriate amount of these compounds in methanol. Acetonitrile (HPLC grade) were purchased from JT Baker (USA), and distilled water was purified "in-house" using a MilliQ system. All samples were filtered through a 0.25-μm pore-size membrane filter to remove the suspended particular matter.

1.2 Instrumentation

Chromatographic separation was performed on a 50 mm × 2.1 mm ACQUITY 1.7 μm column (Milford, Waters Corp., USA) using an ACQUITY Ultra Performance Liquid Chromatograph system. The column was maintained at 40°C and the sample was maintained at 5°C. The flow rate was 0.5 mL/min and was eluted with an isocratic elution of A = water, B = acetonitrile (A:B = 60:40, *V*/*V*). The absorbance wavelength was 200 nm and the injection volume was 10 μL.

Conventional chromatographic separations were performed on a Waters 2487 with Dual λ absorbance detector. The separations were performed on an Xbridge™C18 column (150 × 4.6 mm, particle size, 5 μm, Waters, Ireland). The column was maintained at ambient temperature, the flow rate was 1 mL/min and an isocratic elution of A = water, B = acetonitrile (A:B = 55:45, *V*/*V*) was used. The absorbance wavelength was 200 nm. A 20-μL injection was made into the column.

1.3 Collection of exposure water samples

The exposure of E2 and EE2 to water containing amphioxus (density < 1 g/L), which simulated natural water, was conducted. The beginning nominal levels were set at 1 and 5 μg/L for E2 and EE2, respectively. Water quality parameters included pH (6.9–7.9), oxygen concentration (5–7 mg/L), and temperature (22.5–25.5°C). Sampling was performed during 48 hr exposure. A total of 13 sampling points for E2 and 12 for EE2 were set. At each sampling points, four parallel water samples were collected.

1.4 Sample preparation

Five hundred milliliter exposure water was filtered through a 0.45-μm pore-size membrane filter to remove the suspended particular matter. The C18 cartridges were conditioned with 7 mL acetonitrile, 5 mL methanol, and 5 mL distilled water. Then, 500 mL water samples were extracted at a flow rate of 5–10 mL/min. After the cartridges were washed with 10 mL distilled water, they were dried under a flow of nitrogen for 30 min with the heater at 38°C. The analytes were eluted with 10 mL acetonitrile. The solution was evaporated to dryness under a gentle stream of nitrogen, and redissolved with 0.5 mL acetonitrile, before detection. Then 0.5 mL water was added and the solution was shaken to homogenize, and filtered again with a 0.25-μm pore-size membrane.

2 Results and discussion

2.1 Van Deemter analysis of column efficiency of UPLC

When the four estrogens were separated by UPLC with increased linear velocity, the trends correspond to the van Deemter curves, except for E3 (Fig. 1). In the case of E3, the retention time was very short, with the stationary phase contributing little to E3 when it passed through the chromatogram column. This indicated that the higher the linear velocity, the shorter the retention time. Correspondingly, the smaller the contribution of the stationary phase, the smaller are theoretical plate number (N) and number of effective theoretical plates (Neff).

2.2 Comparison of HPLC and UPLC for the analysis of the four estrogens

Resolution (*R*) is a quantitative measure of the degree of separation between two chromatographic peaks. As shown in Fig. 2, similar resolutions (2.55 for HPLC and 2.65 for UPLC) were obtained for estrogens separated by both HPLC and UPLC.

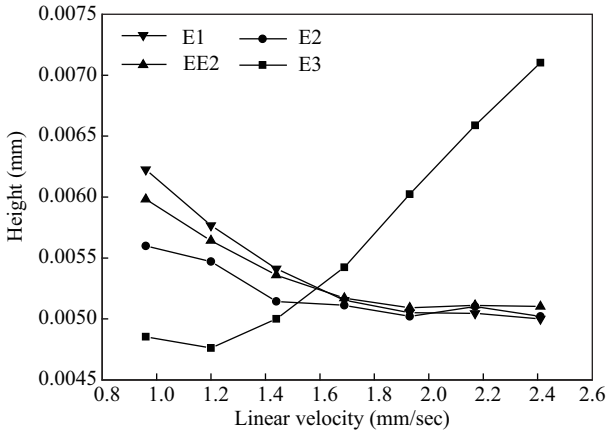

Fig. 1 Relationship curve between linear velocity and height of equivalent to theoretical plates with UPLC^{TM} technology and particle sizes of 1.7 μm stationary phase.

The UPLC methodology provides high sensitivity, with a 1.7-fold increase predicted by chromatographic theory when compared to traditional HPLC (Neue, 1997; Farré et al., 2007; Kong et al., 2010; Jin et al., 2008; Dongre et al., 2008). Table 1 shows the detection limits of the four estrogens generated from HPLC and UPLC under optimum conditions. Obviously, the limit of detection of the UPLC method was two times lower than our HPLC method.

Table 1 Limits of detection of the instrument

Estrogens	Limit of detection (ng/mL)	
	HPLC	UPLC
E3	6.42	2.10
E2	7.01	1.91
EE2	9.52	3.56
E1	7.53	2.51

The most attractive advantage of UPLC is the high separation rate due to the increase in optimal linear velocity. As shown in Fig. 2, it takes less than 2 min to separate the four estrogens using UPLC, compared with 7 min by HPLC. This trend is also seen for balance time, with 1 min needed for UPLC and 5–10 min for HPLC. Using the UPLC method shortens analysis time by up to 10 min compared to our HPLC method and previous literature (Wang et al., 2008).

2.3 Optimization of the separation condition

During the analysis of the environmental water samples after solid phase extraction (SPE), E3 can be interfered by other impurities co-eluted from the SPE cartridges, which leads to inaccurate quantitative analysis. Gradient elution was thus used to let E3 (the first peak in Fig. 3) elute after one minute. Figure 3 shows the optimized chromatogram for the four estrogens when using the following separation programs: 0 min, 0.4 mL/min, 95:5 (water:acetonitrile, *V*/*V*); 3 min, 0.4 mL/min, 60:40 (water:acetonitrile, *V*/*V*), curve: 3; 4 min, 0.4 mL/min, 100% acetonitrile, curve: 1; 5 min, 0.4 mL/min, 95:5 (water:acetonitrile, *V*/*V*), curve: 1. It was clear that successful separation was obtained for the four estrogens without significant interference by impurities under the given conditions.

2.4 Method performance

All standards and samples were injected in triplicate. They were determined by serial dilution of sample solution using the described UPLC conditions. Table 2 shows the

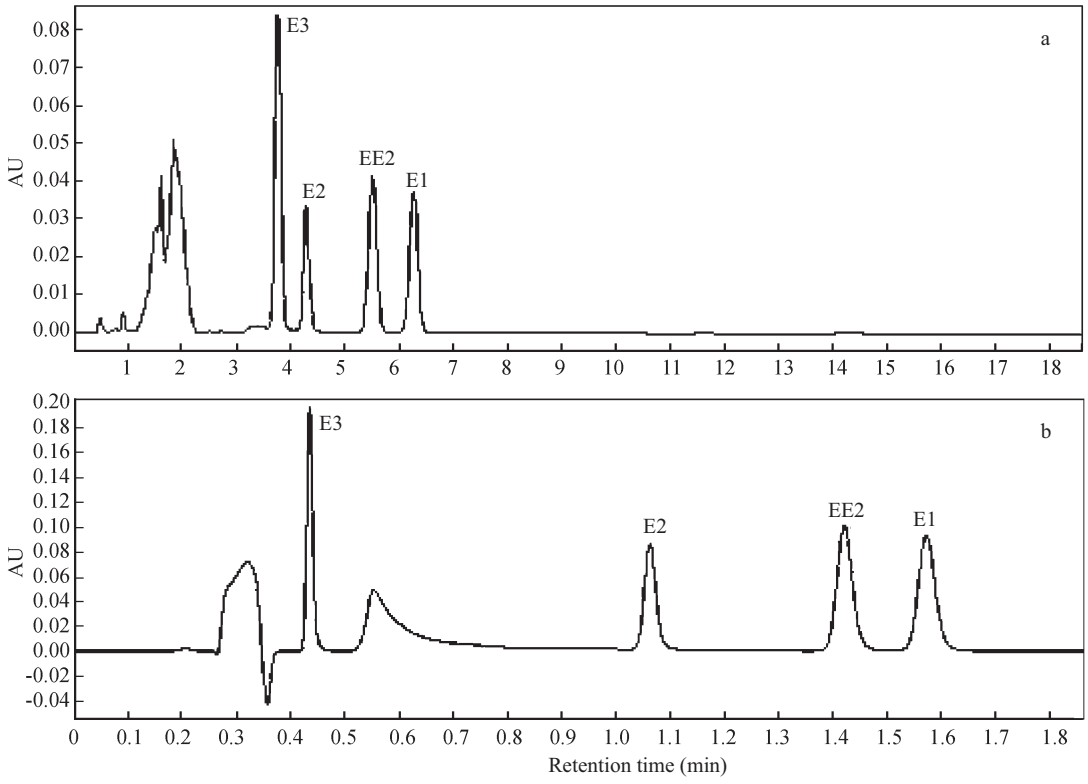

Fig. 2 Chromatograms obtained by analyzing the same estrogens with HPLC (a) and UPLC (b).

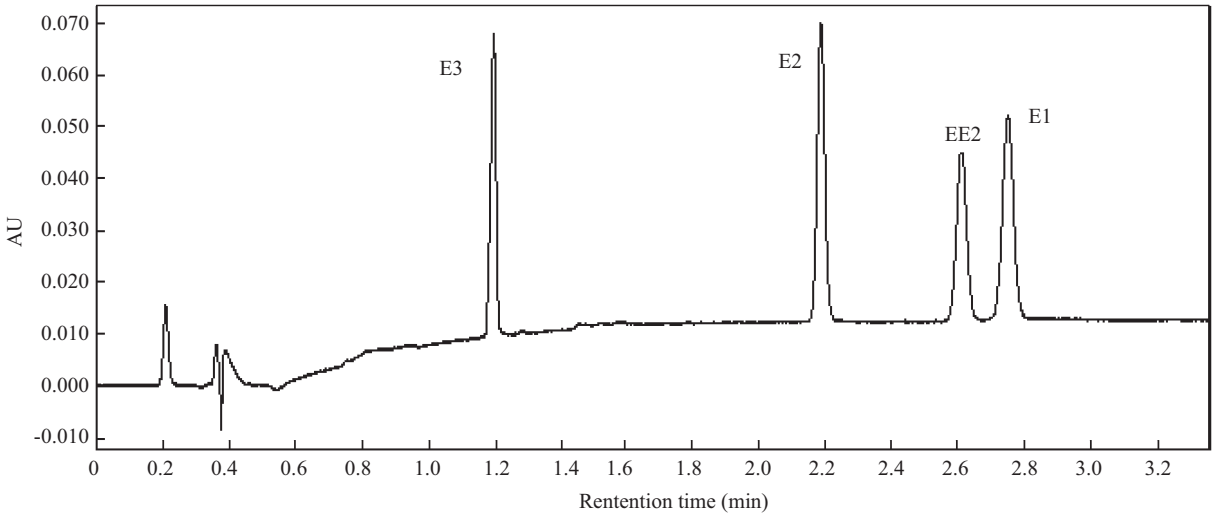

Fig. 3 Chromatogram obtained by analyzing the estrogens under optimum conditions.

Table 2 Analytical characteristics of the method

Analyte	Detection limit (ng/L)[a]	RSD (%)	R^2	Linear range (ng/L)
E3	13.8	6.6	0.9963	20–200
E2	12.5	9.3	0.9990	20–200
E1	16.5	10.6	0.9961	20–200
EE2	23.7	6.6	0.9984	25–200

[a] Limits of detection of the analytical method on the instrument.

detection limits of the analytical method on the instrument for the four estrogens.

The quantitative capability of the system employing the UPLC method was tested in the assay. Each calibration curve was performed with six different concentrations in triplicate. Table 2 shows the results of the standard calibration curves of the integrated peak area (n = 3) and linearity (R^2). Calibration curves were linear with correlation coefficients > 0.999 for all analytes. The results showed excellent correlation between peak area and concentration.

To evaluate the accuracy and reliability of the proposed method, spiked recovery experiments were performed using the exposure water samples spiked with 1 ng/mL E2, EE2 and 5 ng/mL E2, EE2. The mean recoveries of E2 and EE2 were 92% and 90%, respectively. The linear ranges of the method for the four estrogens are also shown in Table 2. The results detailed above confirmed the feasibilities of the actual application of the proposed method.

2.5 Kinetic process of E2/EE2 degradation

Both E2 and EE2 have high potential estrogenic activity. Although their degradation has been reported in many studies (Hashimoto and Murakami, 2009; Suri et al., 2010; Zuo et al., 2006), the degradation process is different due to different backgrounds and conditions, especially for laboratory exposure experiments.

To study their degradation in simulated natural water, E2 and EE2 were dissolved in the water to 1 and 5 ng/mL in different glass tanks, and were irradiated under natural sunlight. Kinetic experiments on the E2 and EE2 degradation processes within 48 hr were completed. First-order kinetics was confirmed by plotting $\ln(C/C_0)$ (C: real-time concentration; C_0: initial concentration) as a function of time to yield a straight line (Fig. 4). The R^2 of first-order fit was in the range of 0.91–0.97, which demonstrated that the degradation was a first-order kinetic process. Moreover, there was a slight difference in the first-order degradation rate between the two different concentration levels. We concluded, therefore, that the first-order degradation rate of E2 and EE2 was irrelevant for initial concentration of estrogens.

Our results differed to previous research (Jürgens et al., 2002), with low levels of E1 found after only several hours and E3 undetected during the entire process. As shown in Fig. 4a, E2 decreased as time passed. After 24 hr about 30% was degraded, with 1 ng/mL E2 decreasing to 0.7 ng/mL and 5 ng/mL E2 decreasing to 3.45 ng/mL. After 48 hr about 70% was degraded and the half-life was about 30 hr, with 1 ng/mL E2 decreasing to 0.28 ng/mL and 5 ng/mL E2 decreasing to 1.53 ng/mL. According to Fig. 4b, as EE2 is somewhat resistant to biodegradation, it decreased less slowly than E2 in the exposure water, which is consistent with other research (Jürgens et al., 2002; Zuo et al., 2006). After 24 hr about 20% was degraded, with 1 ng/mL EE2 decreasing to 0.81 ng/mL and 5 ng/mL EE2 decreasing to 3.38 ng/mL. After 48 hr about 55% EE2 was degraded and the half-life was 46 hr, with 1 ng/mL EE2 decreasing to 0.45 ng/mL and 5 ng/mL EE2 decreasing to 2.45 ng/mL.

Including balance time, the total analytical time using UPLC for one sample was less than 5 min, while HPLC needs more than 10 min. The chromatogram obtained by HPLC was not symmetrical, while the chromatogram obtained by UPLC was relatively symmetrical, which is of benefit for accurate quantitative detection.

3 Conclusions

The UPLC-UV method for the analysis of four estrogens in natural water demonstrated improved performance compared to HPLC-UV. Base-line separation was achieved for all estrogens using a column (50 mm × 2.1 mm) packed with 1.7 μm particle size stationary phase in 1.7 min.

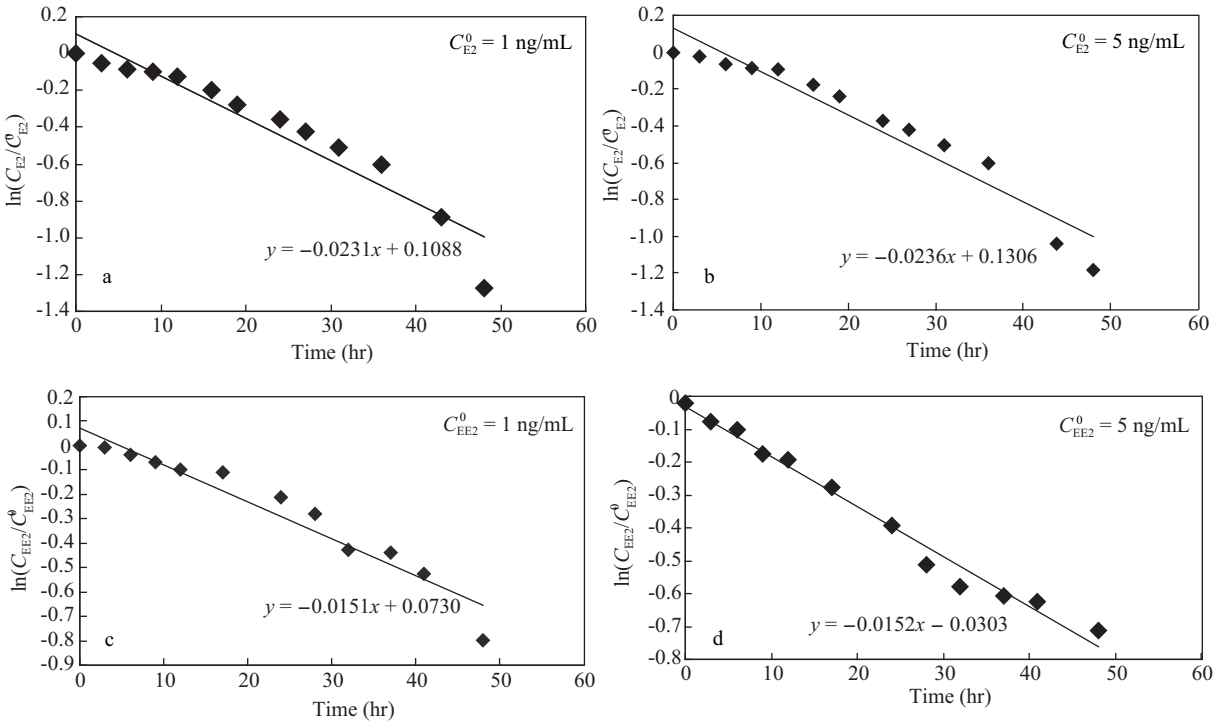

Fig. 4 First kinetic plot for the degradation process for different concentrations of E2 (a, b) and EE2 (c, d) in glass tanks with fish.

Recovery was higher than 88% and detection limits were between 12.5–23.7 ng/L for the four estrogens with the RSD ranging from 7% to 11%. The proposed method was successfully applied to determine changes in E2 and EE2 in simulated natural water with fish in glass tanks. Within 48 hr about 70% E2 had degraded (with a half-life of about 30 hr), whereas about 55% EE2 had degraded (with a half-life of about 36 hr). Low levels of E1 were found, however E3 was undetected during the entire process.

This newly developed UPLC method for all estrogens in natural water gave shorter retention time and maintained better resolution than conventional HPLC. The UPLC method was suitable for rapid analysis of estrogen degradation and was successfully applied to test many samples in a shorter time.

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References

Christensen L J, Korsgaard B, Bjerregaard P, 1999. The effect of 4-nonylphenol on the synthesis of vitellogenin in the flounder *Platichthys flesus*. *Aquatic Toxicology*, 46: 211–219

Coleman H M, Abdullah M I, Eggins B R, Palmer F L, 2005. Photocatalytic degradation of 17β-oestradiol, oestriol and 17α-ethynyloestradiol in water monitored using fluorescence spectroscopy. *Applied Catalysis B: Environmental*, 55: 23–30.

Dongre V G, Karmuse P P, Rao P P, 2008. Kumar development and validation of UPLC method for determination of primaquine phosphate and its impurities. *Journal of Pharmaceutical and Biomedical Analysis*, 46(2): 236–242.

Farré M, Kuster M, Brix R, Rubio F, 2007. Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography-tandem mass spectrometry, and ultra performance liquid chromatography-quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples. *Journal of Chromatography A*, 1160(1-2): 166–175.

Feng X H, Ding S M, Tu J F, Wu F, Deng N S, 2005. Degradation of estrone in aqueous solution by photo-Fenton system. *Science of the Total Environment*, 345: 229–237.

Gillespie D K, Peyster A, 2004. Plasma calcium as a surrogate measure for vitellogenin in fathead minnows (*Pimephales promelas). Ecotoxicology and Environmental Safety*, 58: 90–95.

Hashimoto T, Murakami T, 2009. Removal and degradation characteristics of natural and synthetic estrogens by activated sludge in batch experiments. *Water Research*, 43: 573–582.

Jin Y, Xue X Y, Shi H, Xiao Y S, Zhang F F, Liang X M, 2008. HPLC and UPLC switch for TCM analysis. *World Science and Technology*, 10(1): 80–84.

Jürgens M D, Holthaus K I E, Johnson A C, Smith J J L, 2002. The potential for estradiol and ethinyestradiol degradation in English Rivers. *Environment Toxicology Chemistry*, 21(3): 480–488.

Kong W J, Jin C, Liu W, Xiao X H, Zhao Y L, Li Z L et al., 2010. Development and validation of a UPLC-ELSD method for fast simultaneous determination of five bile acid derivatives in Calculus Bovis and its medicinal preparations. *Food Chemistry*, 120(4): 1193–1200.

Kolpin D, Furlong E, Meyer M, Thurman E M, Zaugg S, Barber L et al., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: A national reconnaissance. *Environmental Science and Technology*, 36(6): 1202–1211.

Korsgaard B, Pedersen K L, 1998. Vitellogenin in *Zoarces*

viviparus: Purification by ELISA and induction by estradiol-17β and 4-nonylphenol. *Comparative Biochemistry and Physiology – Part C*, 120: 159–166.

Liu R, Zhou J L, Wilding A, 2004. Simultaneous determination of endocrine disrupting phenolic compounds and steroids in water by solid-phase extraction-gas chromatography-mass spectrometry. *Journal of Chromatograph*, 1022(1-2): 179–189.

Lv X F, Shao J, Song M Y, 2006. Vitellogenic effects of 17β-estradiol in male Chinese loach (*Misgurnus anguillicaudatus*). *Comparative Biochemistry and Physiology – Part C*, 143(1): 127–133.

López de Alda M J, Barceló D, 2000. Determination of steroids sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography-diode array detection – mass spectrometry. *Journal of Chromatograph A*, 892(1-2): 391–406.

Maria L J, Barceló D, 2001. Review of analytical methods for the determination of estrogens and progestogens in waste waters. *Fresenius' Journal of Analytical Chemistry*, 371: 437–441.

Neue U D, 1997. HPLC Columns: Theory, Technology and Practice. Wiley-VVCH, New York.

Neue U D, Mazzeo J R, 2002. A theoretical study of the optimization of gradients at elevated temperature. *Journal of Separation Science*, 24(12): 921–929.

Patyna P J, Davi R A, Parkerton T F, Brown R P, Copper K R, 1999. A proposed multigeneration protocol for Japanese medaka (*Oryzias latipes*) to evaluate effects of endocrine disruptors. *Science of the Total Environment*, 233(420): 211–220.

Pelly J, 2003. Estrogen knocks out fish in whole-lake experiment. *Environmental Science and Technology*, 37: 313A–314A.

Suri R S, SinghT S, Abburi S, 2010. Influence of alkalinity and salinity on the sonochemical degradation of estrogen hormones in aqueous solution. *Environmental Science and Technology,* 44: 1373–1379.

Turan A, 1996. Excretion of natural and synthetic estrogens and their metabolites: Occurrence and behaviour in water. In Expert Round: Endocrinitically Active Chemicals in the Environment (Gies A, ed.). Umweltbundesamt (German Enviroment Agency), TEXTE 3/96, Berlin.

Verslycke T, Vandenbergh G F, Versonnen B, Arijs K, Janssen C R, 2002. Induction of vitellogenesis in 17α-ethinylestradiol-exposed rainbow trout (*Oncorhynchus mykiss*): A method comparison. *Biochemistry Physiology C*, 132: 483–492.

Wang S, Huang W, Fang G Z, He J X, Zhang Y, 2008. On-line coupling of solid-phase extraction to high-performance liquid chromatography for determination of estrogens in environment. *Analytica Chimica Acta*, 606(2): 194–201.

Wilson I D, Nicholson J K, Castro-Perez J, Granger J H, Johnson K A, Smith B W et al., 2005. High resolution "ultra performance" liquid chromatography coupled to oa-TOF mass spectrometry as a tool for differential metabolic pathway profiling in functional genomic studies. *Journal of Proteome Research*, 4: 591–598.

Ying G G, Kookana R S, Ru Y J, 2002. Occurrence and fate of hormone steroids in the environment. *Environment International*, 28: 545–551.

Zhang K, Zuo Y G, 2005. Pitfalls and solution for simultaneous determination of estrone and 17α-ethinylestradiol by gas chromatography: Mass spectrometry after derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide. *Analytica Chimica Acta*, 554: 190–196.

Zuo Y G, Zhang K, Deng Y W, 2006. Occurrence and photochemical degradation of 17α-ethinylestradiol in Acushnet River Estuary. *Chemosphere,* 63(9): 1583–1590.

Zuo Y G, Zhang K, Lin Y J, 2007. Microwave-accelerated derivatization for the simultaneous gas chromatography-mass spectrometric analysis of natural and synthetic estrogenic steroid hormones. *Journal of Chromatograph* A, 1148: 211–218.