Simultaneous determination of 15 steroidal oral contraceptives in water using solid-phase disk extraction followed by high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid, accurate and sensitive method for simultaneous determination of 15 steroidal hormones including four estrogens (estrone, 17 β -estradiol, 17 α -ethynylestradiol, estriol) and eleven progestogens (17 β -estradiol-3-benzoate, 19-norethindrone, gestodene, levonorgestrel, medroxyprogesterone, cyproterone acetate, megestrol-17-acetate, progesterone, norethindrone acetate, chlormadinone-17-acetate, and hydroxy progesterone caproate) in environmental waters was developed by coupling solid-phase disk extraction (SPDE) to ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) with electrospray ionization. Among three types of extraction tested (C₈ SPDE, C₁₈ SPDE and C₁₈ SPE), the most satisfactory result was achieved using C₁₈ SPDE for its satisfactory recovery (75.6 to 101.4%) and short extraction time (15 min for 1 L deionised water). The validity of this method was investigated and good analytical performance for all the analytes was obtained, including low limits of method detection (0.5–3.4 ng/L) and excellent linear dynamic range (1.0–50.0 ng/L). The method was applied to determine the steroidal hormones in 10 environmental waters including tap water, river water, lake water and waste water in Beijing. No progestogen was detected in all samples and estrone, estriol, 17 α -ethynylestradiol were found in most samples at levels between 1.8 and 127.9 ng/L.

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1. Introduction

The presence of endocrine disrupting chemicals (EDCs) with hormonal activities in the environment has become a major subject of worldwide concern. In many well documented examples the reproduction of wildlife has been adversely affected by EDCs [1–3]. Steroidal hormones, one kind of EDCs, are major part of oral contraceptives which have the potential to exert estrogenic effects in the low ng/L level [2]. The most widely used form of hormonal contraception is the combination of steroidal hormones [4–8]. Those steroidal hormones and their metabolites are constantly excreted into the sewers environment and continue to accumulate in the environment.

For many years, immunoassay methods have been the most sensitive analytical procedures available for the determination of steroidal hormones in biological samples [9–11]. These methods are

sensitive, but prone to cross reactivity by endogenous hormones, co-administrated hormones and their metabolites. Gas chromatography with mass spectrometric (GC/MS) detection has also been the commonly used analytical technology for the determination of soluble steroidal hormones in the past. The limits of detection (LODs) of these methods have been reported to range from 0.005 to 1 ng/L for GC/MS [12–15] and from 0.2 to 40 ng/L for GC/MS/MS [16–18]. But one or multiple steps of derivatization must be included in these methods. Recently, high performance liquid chromatography with mass spectrometric (LC/MS) methods have been favored over GC/MS detection as they do not require a derivatization step prior to analysis. The LODs reported for LC/MS or LC/MS/MS analyses of water-associated hormones range between 0.02 and 1.0 ng/L [19–23]. Accordingly, liquid chromatography with tandem mass spectrometric detection was demonstrated to be superior to immunoassay methods or GC/MS in terms of selectivity, sensitivity, simplicity and analytical throughput.

In order to detect hormones at the ng/L level in waters, sample preparation procedures normally include extraction and enrichment steps. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) [19,21,23] are most common extraction methods used for the determination of steroid hormones. However, LLE

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requires large volumes of organic solvents and additional clean-up steps, and although SPE requires small volumes of organic solvents, the manual version is tedious and time consuming. To overcome these problems, in-tube solid-phase microextraction [20], on-line SPE [22] and stir bar sorptive extraction [12,24] have been developed successfully for the determination of these substances.

Many factors affect the distribution of contraceptive use in different countries or even in different regions of the same country. The current work was focused on the contaminative status of steroidal hormones in Beijing since the use of oral contraceptives in this district. According to the major ingredients in oral contraceptives sold in local drugstores we have selected the 15 steroidal hormones used mostly as the analytes. In the study a method for the simultaneous determination of the selected 15 steroidal hormones (see Fig. 1) in environmental water was developed using solid-phase disk extraction (SPDE) followed with ultra performance liquid chromatography–tandem mass spectrometry (UPLC/MS/MS). This is the first time to use C₁₈ SPDE for the extraction and enrichment of steroidal hormones simultaneously, which reduced sample preparation time within minutes. The effective

method was applied to the 10 environmental waters including tap water, river water, lake water and waste water in Beijing.

2. Experimental

2.1. Chemicals and reagents

The selected steroidal hormones were divided into two groups, group 1 were estrogens and group 2 were progestogens. Standard substance, group 1 (Estrone (E1, purity >99%), 17 β -estradiol (E2, purity >99%), estriol (E3, purity >99%), 17 α -ethynylestradiol (EE2, purity >98.5%)) and group 2 (19-norethindrone (NE, purity >99.5%), gestodene (GES, purity >99%), levonorgestrel (LNG, purity >99%), medroxyprogesterone (MPG, purity >99%), cyproterone acetate (CPA, purity >98%), megestrol acetate (MGA, purity >98.5%), progesterone (PG, purity >98.5%), norethisterone acetate (NEA, purity >99%), chlormadinone acetate (CMA, purity >99%), 17 β -estradiol-3-benzoate (EB, purity >99.5%), hydroxyprogesterone caproate (HPC, purity >98%)), were obtained from Dr. Ehrenstorfer (Augsburg, Germany). HPLC grade solvents of acetonitrile and methanol were

Fig. 1. Molecular structure of the steroidal hormones.

purchased from Burdick and Jackson (Muskegon, MI, USA). Formic acid, acetic acid and ammonium hydroxide were double distilled obtained from GFS Chemicals (Columbus, USA). Ammonium acetate (purity >98%) was purchased from Sigma–Aldrich (Saint Louis, USA). Deionised water was obtained from a Milli-Q Plus 185™ coupled to an Elix 5™ system (Millipore, Schwalbach, Germany). The ENVI-18 (C₁₈), ENVI-8 (C₈) SPE disk (47 mm diameter, 0.6 mm thick) and C₁₈ SPE cartridge (500 mg, 6 mL) were supplied by Supelco (Pennsylvania, USA). Stock standard solutions of individual steroidal hormones (1.0 mg/L) were used to prepare the working and calibration standard mixtures in methanol at the desired concentration, stored refrigerated at –20 °C.

2.2. Sample preparation

Water samples were collected in amber glass bottles from rivers in Beijing and filtered through 0.2 µm nylon membrane which was 47 mm in diameter (Pall, New York, U.S.A). The samples were stored in the dark at 4 °C and then analyzed within 48 h. Since the complicated matrix and solid grain in some environmental water the disk would be jammed and the extraction efficiency would be decreased. To resolve this problem the volume of sample was chosen to be 100 mL which was diluted by 900 mL deionised water before extraction.

To purify the samples, ENVI-18 SPE disk was performed. Initially, disk was washed with 10 mL of methanol followed by 10 mL of deionised water. Afterwards, water sample was drawn into the disk (47 mm diameter, 0.6 mm thick), then dried under a vacuum for 5 min and eluted with 3 × 10 mL of acetonitrile. The obtained extract was evaporated to dryness with rotary evaporator, reconstituted in acetonitrile to a final volume of 0.1 mL and filtered through a 0.2 µm syringe filter which was 13 mm in diameter (Pall, New York, U.S.A) for further UPLC/MS/MS analysis.

C₁₈ cartridge and C₈ disk were also used in the experiment. C₁₈ cartridge was washed with 3 mL of methanol and followed by 3 mL of deionised water. Afterwards, water sample was drawn into the cartridge connected to a vacuum system at the speed of 10 mL/min, then dried under vacuum for 5 min and eluted with 5 mL of acetonitrile. The elution was evaporated to dryness with rotary evaporator, reconstituted in acetonitrile to a final volume of 0.1 mL and filtered for further UPLC/MS/MS analysis. The sample preparation of C₈ disk was the same as the operation of C₁₈ disk.

2.3. UPLC/MS/MS analysis

Chromatographic separation was carried out using a Waters Acquity UPLC system coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass and injection volume was set at 5 µL. Separation was achieved on a Acquity UPLC

Table 1
Retention time, SRM conditions (ESI) for the steroidal hormones by UPLC/MS/MS.

Analyte	Retention time (min)	Precursor ion (<i>m/z</i>)	Product ions (<i>m/z</i>)	Cone (V)	Collision energy (V)
E1	7.48	269.1	144.9 158.9	–60	–38 –37
E2	7.52	271.2	145.0 182.9	–60	–40 –40
E3	2.04	287.1	144.9 170.9	–60	–40 –35
EE2	7.68	295.1	144.9 159.0	–60	–40 –35
NE	3.63	299.1	231.1 281.1	35	18 17
GES	3.95	311.1	108.9 293.1	35	26 15
LNG	4.39	313.1	109.0 245.1	35	27 18
MPG	4.76	345.1	122.9 327.2	35	24 17
CPA	5.39	417.0	321.2 357.0	35	17 17
MGA	5.44	385.3	267.1 282.2	35	30 30
PG	5.45	315.3	109.0 123.0	35	25 30
NEA	5.56	341.1	281.1 299.1	35	17 17
CMA	5.64	405.3	267.1 301.1	35	30 28
EB	7.57	377.3	104.9 134.9	35	35 35
HPC	7.60	429.3	271.1 313.2	35	19 15

BEH RP-C₁₈ (50 × 2.1 mm, 1.7 μm) preceded by a guard column (4 × 4 mm, 1.7 μm) of the same packing material from Waters (Ireland, U.S.A). The flow rate of mobile phase was 0.1 mL/min under isocratic conditions and the column temperature was set at 30 °C. The chromatography was carried out using mobile phases A (methanol for group 1 and acetonitrile for group 2) and B (deionised water) and the separation was performed using a gradient profile. The operating conditions of the UPLC for group 1 were: 50% A at initial time, and change to 100% A in 5 min, keep 100% A for 5 min. For group 2 the gradient profile was: 30% A at initial time, and change to 100% A in 10 min. At the end of the chromatogram, the mobile phase was changed to its initial composition and the column was equilibrated.

Ionization and fragmentation settings were optimized by direct injection of standard solutions. MS/MS was performed in the selected reaction monitoring (SRM) using ESI in the negative ion mode for group 1 and positive ion mode for group 2. For each compound, two characteristic fragmentations of the molecular ion were monitored (Table 1), the second one being used for quantification, while the first one was used as a qualifier. Collision energy and cone voltage were optimized for each analytes. Other parameters were optimized as follows: capillary voltage –3.5 kV for group 1 and 3.3 kV for group 2, extractor voltage –4V for group 1 and 3 V for group 2, multiplier voltage 650 V, source temperature 100 °C, desolvation temperature 450 °C. Argon was used as collision gas (P collision cell: 3×10^{-3} mbar), while nitrogen was used as both the nebulizing (50 L/h) and desolvation gas (700 L/h). Data was acquired using MassLynx 4.0.

2.4. Data analysis

Identification of the analytes was performed by comparing the retention times and the MS signals in the samples with those obtained using standard solutions. Furthermore, for UPLC/MS/MS analyses, the ratio of the two precursor-product ion transitions was compared to that observed for authentic standard (using a tolerance of ±30%). Quantification was carried out by calculating the response of quantitative ion. Concentrations were determined using linear regression analysis of the peak area. This six point calibration curve ($R^2 > 0.99$) using the external standard methodology covered the range 1–50 ng/L of the standard solutions.

3. Results and discussion

3.1. Optimization of SPDE conditions

SPE was the most common extraction method used for the isolation of hormones from water and always showed excellent extraction efficiency. To develop a fast method for the treatment of large volume sample, SPE disk was chosen which could treat 1 L water in minutes [25,26,19]. In the work C₁₈ SPE cartridge, ENVI-18 SPE disk and ENVI-8 SPE disk were tested for the sample preparation. All the experiments were performed by spiking with standard solutions at 50 ng/L to 1 L deionised water in this section. The recoveries of C₁₈ cartridge, C₁₈ disk and C₈ disk were 74.1–99.8, 76.7–97.3, 23.9–78.9 respectively shown in Table 2. According to the data, the extraction efficiency and repeatability obtained with the C₁₈ disk are similar to those obtained with C₁₈ cartridge, but the sample preparation time of the former was drastically reduced. It took 20 min in the extraction with C₁₈ disk whereas 2 h at least using C₁₈ cartridge. C₈ disk failed to retain all the analytes although with good repeatability. So C₁₈ disk was the best choice for sample preparation within minutes.

To optimize the extraction speed, extraction recovery experiments were performed by spiking with the standard solutions at 50 ng/L into 1 L deionised water at the extraction speed of 50,

Table 2

Recoveries (%) of the steroidal hormones spiked at 50 ng/L to 1 L deionised water by use of C₁₈ cartridge and C₁₈, C₈ disk ($n = 5$).

Analyte	C ₁₈ cartridge	C ₁₈ disk	C ₈ disk
E1	87.9 ± 1.2	90.7 ± 1.3	54.2 ± 3.4
E2	99.8 ± 2.4	90.4 ± 4.0	42.3 ± 0.9
E3	96.0 ± 2.5	91.3 ± 1.4	72.6 ± 6.1
EE2	92.2 ± 2.3	93.1 ± 1.4	51.3 ± 1.6
NE	92.9 ± 5.6	89.1 ± 4.6	77.3 ± 0.4
GES	86.3 ± 2.6	81.7 ± 5.3	78.9 ± 7.1
LNG	82.2 ± 3.6	84.2 ± 6.2	60.0 ± 3.4
MPG	80.7 ± 5.3	95.0 ± 9.4	51.7 ± 0.2
CPA	83.3 ± 1.1	91.8 ± 6.1	56.3 ± 4.1
MGA	80.9 ± 1.4	89.1 ± 8.9	50.0 ± 5.3
PG	81.7 ± 0.8	97.3 ± 9.2	65.4 ± 4.4
NEA	83.2 ± 8.0	87.8 ± 6.9	51.7 ± 9.1
CMA	92.8 ± 2.9	94.9 ± 6.0	62.1 ± 5.2
EB	78.8 ± 0.4	86.8 ± 0.8	45.0 ± 4.9
HPC	74.1 ± 1.2	76.7 ± 8.4	23.9 ± 2.4

100, 150 mL/min. According to the result the extraction efficiency changed little at the different speed and the SPE disk extraction speed of 150 mL/min was chosen for sample preparation in our experiment. Another important parameter was elution solvent. In this work, elution solvent was optimized by spiking with the standard solutions at 50 ng/L to 1 L deionised water. According to the characters of steroidal hormones four elution solvents, acetonitrile, ethyl acetate, methanol and dichloromethane, were tested. Fig. 2 showed that acetonitrile was the best elution solvent for the most analytes except E2 and E3 and the recoveries of that were no less than 83.7%. While ethyl acetate was used as the elution solvent, the recoveries were very low, between 14.1% and 76.4% for the 15 steroidal hormones.

3.2. Optimization of the mobile phase for UPLC–MS/MS

It was reported that the suppression or enhancement effects of matrix might be caused by polar, non-retained matrix components and also depend on the mobile phase, nature of individual sample matrix and ionization source used [9,18,22,27]. In the study, the attempts to increase response with different mobile phase were done under the MS conditions used. For group 1 E2 was chosen to optimize the mobile phase because of the similar characters of four estrogens and for group 2 LN and MGA were chosen since they had the same representative structure of the 11 progestogens. Six mobile phases were tested by the standard solutions at 50 ng/L and the data was shown in Table 3. According to the data we can conclude that the best response was obtained of the mobile phase consisting of methanol–water for group 1 and acetonitrile–water for group 2.

3.3. Linearity

Quantification was performed using an external six point calibration curve covering the range from 1 to 50 ng/L and the curves were obtained by plotting the peak area ratio of the analytes. Excellent linearity was achieved in these specified concentration ranges with the correlation coefficients higher than 0.9934 for all validation batches. The calibration curves obtained as described above were suitable for the quantification of all the analytes in the water sample during the intra- and inter-day validations and stability tests. The method detection limits (MDL) for 15 steroidal hormones listed in Table 4 were 0.5–1.2 ng/L for group 1 and 0.8–3.4 ng/L for group 2 in waste water.

3.4. Repeatability and precision

The intra-assay recoveries of the method were determined by analyzing deionised water, tap water and waste water spiked

Fig. 2. Effects of the different elution solvents. Each deionised water sample was spiked with steroidal hormones at 50 ng/L and extraction speed was 150 mL/min.

Table 3

Concentrations of analytes using different mobile phase in UPLC.

Mobile phase	Concentration (ng/L)	Mobile phase	Concentration (ng/L)
	E2		LNG
Acetonitrile–water	30.3	Acetonitrile–water	50.0
Acetonitrile–ammonia aqueous	10.1	Acetonitrile–formic acid aqueous	28.2
Acetonitrile–ammonia acetate aqueous	0.7	Acetonitrile–acetic acid aqueous	28.5
Methanol–water	50.0	Methanol–water	37.9
Methanol–ammonia aqueous	16.9	Methanol–formic acid aqueous	15.7
Methanol–ammonia acetate aqueous	1.5	Methanol–acetic acid aqueous	18.8
			MGA
			50.0
			36.6
			41.3
			42.5
			16.9
			15.3

with steroidal hormones at 10 and 50 ng/L respectively. SRM chromatograms were shown in Fig. 3. The accuracy of the method was determined by calculating recovery and the precision was determined by calculating the relative standard deviation (RSD). Table 4 summarized the recoveries of each assays for 15 steroidal hormones in various water matrices. The recoveries were 75.6–101.4% for deionised water, 82.2–102.1% for tap water and 77.3–93.0% for waste water when the samples were spiked at 10 ng/L standard solutions and the RSD were 0.4–2.3% for deionised water, 0.9–3.8% for tap water and 0.6–7.4% for waste water. The recoveries and RSD of samples spiked at 50 ng/L standard solutions were similar to the former.

3.5. Application to real samples

The proposed UPLC/MS/MS method was successfully applied to the analyses of the 15 steroidal hormones in 10 environmental waters commonly found in Beijing area. The sampling site of water and concentration of steroidal hormones in samples were

shown in Table 5. Group 2 were not detected in all samples and E1, E3, EE2 were detected in most samples. In these samples, E1 was detected in five samples that the concentration was from 1.8 ± 0.7 to 3.9 ± 0.9 ng/L and E3 was detected only in sample 5 at 13.9 ± 1.2 ng/L. EE2 was detected in all the environmental samples except tap water and the concentration was from 5.0 ± 1.3 to 127.9 ± 2.6 ng/L (Fig. 4). The results obtained in my work were similar with previously published data [21,28]. Comparing the concentrations of natural steroidal hormones (E1, E3) with that of synthetic steroid hormone (EE2) in environmental samples we found the latter was much higher not only in river water but also in lake water. EE2 has been ordinary ingredient of oral contraceptives sold in Beijing. On the other hand EE2 have been used extensively therapeutic purposed, for example management of menopausal syndrome and in a wide range of cancers [29,30]. And the amount of EE2 in those medicines was high and for such reason the residual amount of EE2 became higher in environmental water. In China, different progestogens have been used dispersedly in various brand contraceptives except that EE2 was often the assistant ingredient.

Table 4

Recoveries of the steroidal hormones spiked with standard solutions in various matrices ($n = 5$) and method detection limits (MDL) in waste water.

Analyte	Recovery (%)						MDL (ng/L)
	Spiked 10 ng/L			Spiked 50 ng/L			
	Deionised water	Tap water	Waste water	Ultrapure water	Tap water	Waste water	
E1	88.0 ± 1.2	82.2 ± 1.5	83.0 ± 1.9	90.7 ± 1.3	84.7 ± 7.4	88.2 ± 3.8	0.5
E2	93.2 ± 1.5	92.9 ± 2.4	91.8 ± 0.8	90.4 ± 4.0	100.6 ± 9.0	94.9 ± 4.7	0.6
E3	88.4 ± 1.0	90.4 ± 1.3	86.7 ± 0.6	91.3 ± 1.4	98.0 ± 6.3	93.4 ± 2.0	1.0
EE2	90.6 ± 1.3	86.9 ± 1.8	88.8 ± 1.3	93.1 ± 1.4	82.7 ± 10.4	88.9 ± 3.9	1.2
NE	97.0 ± 0.4	89.2 ± 0.9	76.4 ± 1.5	89.1 ± 4.6	78.4 ± 7.8	98.2 ± 5.3	2.8
GES	86.3 ± 1.2	81.6 ± 2.5	86.0 ± 2.8	81.7 ± 5.3	84.3 ± 9.4	96.6 ± 10.8	3.4
LNG	95.7 ± 1.1	95.2 ± 3.1	64.2 ± 1.6	84.2 ± 6.2	87.6 ± 8.8	85.8 ± 4.1	0.8
MPG	100.3 ± 1.0	97.4 ± 2.2	92.7 ± 7.4	95.0 ± 9.4	93.5 ± 5.9	96.4 ± 5.6	1.0
CPA	87.7 ± 1.4	88.9 ± 1.9	84.6 ± 3.7	91.8 ± 6.1	89.2 ± 11.7	86.9 ± 7.0	0.9
MGA	89.3 ± 2.0	89.2 ± 3.1	79.4 ± 6.4	89.1 ± 8.9	89.6 ± 3.5	88.2 ± 4.4	0.9
PG	96.7 ± 0.9	92.6 ± 1.7	89.5 ± 2.6	97.3 ± 9.2	80.2 ± 4.8	90.6 ± 5.1	0.8
NEA	101.4 ± 0.4	102.1 ± 0.9	84.5 ± 1.5	87.8 ± 6.9	91.7 ± 8.6	86.1 ± 11.1	0.8
CMA	99.7 ± 0.6	97.2 ± 1.3	93.0 ± 3.0	94.9 ± 6.0	91.2 ± 5.0	93.2 ± 4.6	1.0
EB	83.8 ± 1.1	86.6 ± 2.4	84.7 ± 2.1	86.8 ± 0.8	87.7 ± 12.4	86.1 ± 8.5	1.3
HPC	75.6 ± 2.3	82.4 ± 3.8	77.3 ± 6.2	76.7 ± 8.4	89.9 ± 6.8	81.8 ± 7.4	1.8

Fig. 3. SRM chromatograms corresponding to a steroidal hormones standard solution of 50 ng/mL. For chromatographic and instrumental conditions, see Section 2.3.

Table 5Concentrations of the steroidal hormones in environmental water samples taken at 10 different sampling sites in Beijing ($n = 3$).

Sample number	Sample type	Sampling site	Concentrations of steroidal hormones (ng/L)		
			E1	E3	EE2
1	Tap water	Lab	–	–	–
2	Waste water	Gaobeidian	3.9 ± 0.9	–	49.3 ± 1.4
3	Treated sewage	Gaobeidian	3.0 ± 1.0	–	46.7 ± 1.4
4	River water	Tonghui river (Dawang Road)	–	–	32.9 ± 5.2
5	River water	Tonghui river (Bali Bridge)	–	13.9 ± 1.2	68.8 ± 5.5
6	River water	Wenyu River	1.8 ± 0.7	–	26.1 ± 5.9
7	River water	Liangshui River	1.8 ± 0.9	–	74.6 ± 4.8
8	River water	Tongzhou Canal	2.3 ± 0.9	–	127.9 ± 2.6
9	River water	Tsinghua University	–	–	5.0 ± 1.3
10	Lake water	Kunming Lake	–	–	8.5 ± 1.6

–: Not determined.

Fig. 4. SRM chromatograms of (A) E1 in sample 2 (B) E3 in sample 5 (C) EE2 in sample 7 obtained from the analysis of environmental water samples.

Hence those progestogens have not been detected in the samples because of low concentration. As some researches reported E2 is easy to be oxidized into E1 under biotic and abiotic degradation [31–33] so we have not found it in these environmental samples.

4. Conclusions

A method based on solid-phase disk extraction coupled with UPLC/MS/MS analysis has been developed for simultaneous determination of 15 steroidal hormones in water. It was important to use SPE disk to treat large volume water sample in short sample preparation time, only minutes. This new method is an improvement in terms of simplicity and efficiency which can be applied

for their validity in steroidal hormones in environment studies. In this work 10 water samples including tap, river, lake and waste waters were detected. No progestogen was detected in all samples and E1, E3, EE2 were detected in the most samples. In these samples, E1 was detected in five samples that the concentration was from 1.8 ± 0.7 to 3.9 ± 0.9 ng/L and E3 was detected only in sample 5 at 13.9 ± 1.2 ng/L. EE2 was detected in all the environmental samples except tap water that the concentration was from 5.0 ± 1.3 to 127.9 ± 2.6 ng/L. According to this data, the conclusion is that the contaminant of EE2 was serious in Beijing district could be drawn.

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