Dispersive liquid-liquid microextraction (DLLME) coupled with CE was successfully developed for the simultaneous determination of four types of phenolic environmental estrogens (PEEs), namely hexestrol (HS), bisphenol A (BPA), diethylstilbestrol (DES) and dienestrol (DS). Several parameters affecting DLLME and CE conditions were systematically investigated including the type and volume of extraction solvent and dispersive solvent, extraction time, salt, pH value, surfactant, buffer solution and so on. Under the optimal conditions, DLLME-CE exhibited strong enrichment ability, presenting high enrichment factors of 467, 241, 367 and 362 for HS, BPA, DES and DS, respectively, as well as low detection limits of 0.3, 0.6, 0.6 and 0.3 μg/L, respectively. Excellent linearity was achieved in the range of 2.0–150 μg/L for HS and DS, and 4.0–300 μg/L for BPA and DES, with correlation coefficients $R^2$>0.9983. Recoveries ranging from 70.4 to 108.1% were obtained with tap water, lake water and seawater samples spiked at three concentration levels and the relative standard deviations (RSDs, for $n=5$) were 2.1–9.7%. This DLLME-CE method with high selectivity and sensitivity, high stability, simplicity, cost-effectiveness, eco-friendliness was proved potentially applicable for the rapid and simultaneous determination of PEEs in complicated water samples.

Keywords:
Capillary electrophoresis / Dispersive liquid-liquid microextraction / Phenolic environmental estrogens / Water samples

DOI 10.1002/elps.201500519
Recently, estrogens detection has attracted serious attention. Various analytical techniques have been developed to detect various matrixes of estrogens, including HPLC [9–11], liquid chromatography-mass spectrometry (LC-MS) [12, 13], GC [14], CE [15, 16], CE coupled to mass spectrometry (CE-MS) [17, 18], immunoassay [19] and so on. CE has the advantages of low solvent consumption, high separation efficiency, automatic operation and it has been developing quickly [15–18, [20]]. MEKC, one mode of CE, attributes its migration principle to electrophoresis and its separation principle to chromatography, and it enables the application range of CE to be expanded to neutral compounds [21, 22].

On the other hand, considering their low concentration presence in complicated matrices, a number of pretreatment approaches have been developed to concentrate the estrogens and decontaminate environmental samples. For examples, liquid-liquid extraction [17, 23] SPE [24, 25], solid-phase microextraction (SPME) [26, 27], stir bar sorptive extraction [28] and cloud-point extraction (CPE) [29] have been reported for estrogens. However, the classical extraction methods, liquid-liquid extraction and SPE, require large volumes of organic solvents and long preparation time. Dispersive liquid-liquid microextraction (DLLME) first introduced in 2006 [30] has the advantages of simplicity, rapidity and high enrichment ability, which shows a wide application prospect in the analysis of organic compounds and metal ions, and even in the speciation analysis of trace elements [31].

In this work, the four types of estrogens including HS, BPA, DES and DS in water samples were simultaneously concentrated, separated and detected using DLLME coupled with CE. The method demonstrated good analytical performances such as wide linear range, good reproducibility, low detection limit and high recovery. It was expected to provide an alternative for simultaneous determination of trace estrogens in water samples.

2 Materials and methods

2.1 Reagents and samples

Four PEEs standards of HS, BPA, DES and DS were purchased from Sigma-Aldrich (Shanghai, China), and appropriate amounts were dissolved in methanol at a concentration of 1000 mg/L, respectively. SDS and carbon tetrachloride were purchased from Aladdin (Shanghai, China). Chromatographic grade acetonitrile, methanol, ethanol and chlorobenzene were all purchased from J&K Chemical (Beijing, China). The other chemicals, such as sodium hydroxide, sodium tetraborate decahydrate (Na$_2$B$_4$O$_7$·10H$_2$O), acetone, carbon dichloride and chloroform were of analytical grade and were obtained from Sinopharm Chemical Reagent (Shanghai, China). Working standard solutions were prepared daily by diluting the stock solutions to the required concentrations using methanol and all standard solutions were stored in a refrigerator at 4°C for use. The water used throughout the work was produced by a Milli-Q ultrapure water system (Millipore, Bedford, MA, USA).

Tap water samples were collected when needed from our laboratory after it had flowed for about 5 min. Lake water samples were collected into a teflon bottle from an artificial lake named Sanyuan Lake located in the schoolyard of Yantai University. Surface seawater samples were collected in seawater side to the east gate of Yantai University. All water samples were filtered through microporous nylon filters with a pore diameter of 0.45 μm before use and protected from light.

2.2 Apparatus and CE conditions

A P/ACE MDQ CE system (Beckman Coulter, Fullerton, CA, USA) in conjunction with a DAD was used to separate the estrogens. Bare fused-silica capillaries, with id of 75 μm, od of 375 μm, total length of 50.2 cm and effective length of 40 cm (Yongnian Photoconductive Fiber Factory, Hebei, China), were used throughout the whole experiment. The pH of the buffer solution was calibrated by a pHS-3C digital pH meter (Hangzhou Dongxing Instrument Factory, Hangzhou, China). A new capillary was initialized by rinsing with water for 10 min, 1.0 M NaOH for 40 min, water for 10 min and running buffer for 30 min before use. The capillary was rinsed with running buffer for 3 min between analyses. All solutions were filtered through microporous nylon filters with a pore diameter of 0.45 μm before use. The detection wavelength was set at 228 nm. The injection pressure was applied at 0.5 psi for 5 s and separation voltage at 28 kV. The running buffer consisted of 10 mM Na$_2$B$_4$O$_7$·10H$_2$O, 20 mM SDS, and 40% (v/v) acetonitrile, adjusted to pH 10.8 with 1 M NaOH.

2.3 DLLME procedure

For the DLLME, 10 mL aqueous sample solution containing HS, BPA, DES and DS was placed into a 15 mL centrifuge tube with conical bottom. The mixture of extraction solvent (chlorobenzene, 80 μL) and disperser solvent (acetone, 1000 μL) was quickly injected into the aqueous sample using a syringe and then the mixture was gently shaken with hand. Chlorobenzene was dispersed throughout the aqueous phase and a cloudy solution of fine droplets was formed. After the cloudy solution was centrifuged for 5 min at 5000 rpm, the extracting solvent containing the analytes was sedimented at the bottom of the tube. The supernatant liquid was removed and deserted, and the sedimentation phase was transferred to a smaller-sized centrifuge tube followed by drying under a gentle flow of nitrogen. Finally, the residue was redissolved with 20 μL methanol for further CE analysis. The schematic illustration of the DLLME-CE procedure is shown in Supporting Information Fig. S1.

2.4 Enrichment factor

Enrichment factor (EF) was used to evaluate the extraction efficiency, and was calculated as the ratio of $C_{ed}$ to $C_0$, where $C_{ed}$ is the concentration of analyte in the sedimented phase,
and $C_0$ is the initial concentration of analyte in the aqueous solution.

3 Results and discussion

3.1 Optimization of CE conditions

Initially, we purposed to use MEKC mode for the separation of the four PEEs by referring to the previous work [29, 32]. Factors affecting separation were optimized including running buffer, surfactant, organic modifier and applied voltage. Firstly, we investigated two types of buffer solutions, i.e., $\text{Na}_2\text{B}_4\text{O}_7\cdot10\text{H}_2\text{O}$ and $\text{NaH}_2\text{PO}_4$. Result showed that the chromatographic peaks of the four PEEs were deteriorated, let alone baseline separation in the $\text{NaH}_2\text{PO}_4$ solutions. Interestingly, $\text{Na}_2\text{B}_4\text{O}_7\cdot10\text{H}_2\text{O}$ presented better peak shape and resolution. Consequently, different concentrations of $\text{Na}_2\text{B}_4\text{O}_7\cdot10\text{H}_2\text{O}$ (5, 10, 15, 20, 30 mM) were optimized. 10 mM $\text{Na}_2\text{B}_4\text{O}_7\cdot10\text{H}_2\text{O}$ offered higher peak height than that of 5 mM, and the four PEEs were not separated well as the concentrations exceeded 10 mM. According to our experiences and related literatrues, SDS was employed as surfactant micelle. The concentrations of SDS (10, 15, 20, 25, 30 mM) were tested. As shown in Supporting Information Fig. S2, better peak shape and higher resolution could be obtained when SDS was at 20 mM, which was also time-saving than that of 30 mM with base-line separation. So, the MEKC was established by using the solution composing of 10 mM $\text{Na}_2\text{B}_4\text{O}_7\cdot10\text{H}_2\text{O}$ and 20 mM SDS.

The buffer pH is also one important parameter for separation because it can affect the ionization of analytes and further alter their electrophoretic mobility [33]. The pH values of 10.70, 10.75, 10.80, 10.85 and 10.90 were investigated, which were adjusted by 1.0 M NaOH. As shown in Supporting Information Fig. S3, the larger peak-height value was achieved with the pH value of 10.80, while at pH of 10.90, the peaks of BPA and DES could not be well resolved. Thus, the buffer pH was adjusted to 10.80 for the following work.

The addition of organic modifier to the MEKC buffers was an effective way of improving separation selectivity, efficiency and resolution. We selected acetonitrile as the organic modifier in MEKC for its superior electroosmotic flow (EOF)-promoting ability [20]. The proportion of acetonitrile (30, 35, 40, 45, 50% (v/v)) was investigated. Related electrophoretograms were shown in Supporting Information Fig. S4. As observed, 40% (v/v) ACN provided more smooth peak shape with higher resolution and relatively short separation time. Hence, 40% (v/v) ACN was chosen, although the critical micelle concentration of SDS is higher than 30 mM for a volume fraction of ACN of 40% [34]. However, if no SDS was added, the four PEEs could not be separated without EOF; and a higher concentration of SDS caused longer migration time and distorted peaks. Although it is hardly possible to form conventional micelles for the 20 mM SDS in the 40% ACN solution [34], the appropriate concentration SDS (20 mM) played an important role for...
3.2.1 Selection of extraction solvent

In DLLME, a good extraction solvent should have the following characteristics: density higher than water, high extraction capability of the interested analytes, low solubility in water and good chromatographic behavior [36]. Therefore, CH₂Cl₂, CHCl₃, CCl₄ and C₆H₅Cl were selected as extraction solvents, with acetone as dispersive solvent. The results showed aqueous phase and organic phase were mutual after centrifugation when CH₃Cl₂ and CHCl₃ were used as extraction solvents. C₆H₅Cl and CCl₄ had the extraction ability for the four PEEs. And C₆H₅Cl had obviously superior extraction efficiency to BPA compared with CCl₄. So, C₆H₅Cl was selected as the extraction solvent.

3.2.2 Selection of dispersive solvent

The dispersive solvent should meet the requirement of the good miscibility in both aqueous phase and extraction solvent, and then it enables the dispersion of fine droplets of the extractant into the aqueous phase containing the analytes. Based on the above considerations, methanol, ethanol, acetonitrile and acetone were examined. The results showed that acetone had the better enrichment effect than the others especially for BPA. Therefore, acetone was chosen as dispersive solvent.

3.2.3 Selection of the volume of extraction solvent

To examine the effect of extraction solvent volume on the extraction efficiency, the experiments were performed by using 0.8 mL acetone containing different volumes of C₆H₅Cl (40, 50, 60, 70, 80, 90 μL). As shown in Fig. 1A, the extraction efficiency increased with the increase of extracting volume from 40 to 80 μL, and then decreased with further increasing extracting volume. Hence, 80 μL C₆H₅Cl was chosen as extraction solvent for the following studies.

3.2.4 Selection of the volume of dispersive solvent

The optimized DLLME conditions, the volume of dispersive solvent was also investigated by using different volumes of acetone (0.4, 0.6, 0.8, 1.0, 1.2 mL) with the fixed volumes of C₆H₅Cl (80 μL). As shown in Fig. 1B, the extraction efficiency was the maximum when the volume of acetone was 1.0 mL. This was mainly because that, in the lower volumes of acetone, C₆H₅Cl was not dispersed well and the cloudy state was not formed well, so the extraction efficiency was relatively low. The solubility of the analytes in the aqueous phase was increased when higher volumes acetone was added. So, 1.0 mL of acetone was chosen as the optimum volume.

3.2.5 Effect of extraction time

In DLLME, extraction time is referred to the interval time, between the injection of the mixture of extraction solvent (C₆H₅Cl) and dispersive solvent (acetone), and the time starting to centrifuge. The time from 0 to 30 min was investigated. As is shown in Fig. 1C, extraction time had no
Table 1. Comparison of LOD and EF for PEEs determination using DLLME-CE with other CE and HPLC methods

| Detection technique | Pretreatment technique | LOD (µg/L) | EF | Extraction time (min) | Separation time (min) | Ref. |
|---------------------|------------------------|------------|----|-----------------------|----------------------|-----|
| CE-DAD DLLME       |                        | 0.3, 0.6, 0.6, 0.3 | 467, 241, 367, 362 | A few seconds         | 7                     | This work |
| CE-UV dCPE         |                        | 7.9, 8.9, 8.2 | 50–150 | 40                     | 7                     | [29] |
| HPLC-UV dSPE       |                        | 3.0, 6.1, 0.2 | –     | 40                     | 8                     | [37] |
| HPLC-DAD UA-CPE    |                        | 0.1, 0.2, 0.1 | 78.39, 94.34, 86.59 | 45                     | 11                    | [10] |
| HPLC-UV DLLME      |                        | 0.07        | –     | A few seconds         | 4                     | [11] |
| HPLC-UV MMIPs–SPME |                        | 1.5–5.5     | –     | 20                     | 14                    | [27] |
| HPLC-UV DLLME      |                        | 0.010, 0.010, 0.008 | 71, 78.5, 73.5 | A few seconds         | 10                    | [35] |

a) Four analytes are HS, BPA, DES and DS, respectively.
b) Dual cloud point extraction. Three analytes are HS, DES and DS, respectively.
c) Dispersive SPE. Analytes are BPA, DES and DS, respectively.
d) Ultrasound-assisted cloud-point extraction. Analytes are 17β-estradiol (E2), estrone (E1) and DES.
e) Analyte is BPA.
f) Solid-phase microextraction method based on magnetic molecularly imprinted polymer as fiber coating. Analytes are E1, E2, estriol (E3) and DES.
g) Analytes are E1, E2 and DES.

Figure 3. Electrophoregrams of lake water samples after DLLME without spiking (A), and spiked with HS, BPA, DES and DS at 25, 50, 50 and 25 µg/L, respectively, (B) and spiked with HS, BPA, DES and DS at 100, 200, 200 and 100 µg/L, respectively, (C). Peaks: (1) HS, (2) BPA, (3) DES and (4) DS. CE conditions were the same as described in Fig. 1.

significant effect on extraction efficiency at the initial 10 min. The reason is that the surface area between the extractant solvent and the aqueous phase is infinitely large and could equilibrate very quickly. The peak height slightly decreased when the time exceeded 10 min (Fig. 1C) mainly because the PEEs were dissolved in the water samples again. Therefore, centrifugation should be immediately performed after a cloudy solution of fine droplets was formed. That is, extraction time is quite short (usually within a few seconds), which is exactly a major advantage of DLLME.

3.2.6 Effect of salt

The effect of salt on the extraction efficiency was evaluated by adding NaCl (0–5%, w/v) into the aqueous solution. That is, the addition of 1, 2, 3, 4 and 5% NaCl was examined, respectively. When the concentration was lower than 3%, it was observed that the salt did not have significant effects on peak shape, separation resolution and reproducibility, and only extraction efficiency was slightly increased according to peak height calculation, owing to slightly increased solubility of PEEs. When the concentration increased from 3–5%, the peak shape, separation resolution and reproducibility became undesirable, as well as peak height decreased leading to decreased extraction efficiency, which may be because the solubility of PEEs decreased by increasing salt concentration. Considering the slight difference, for simplicity, hence, NaCl was not added in the subsequent experiments.

In short, the DLLME conditions were optimized, i.e., C₆H₅Cl as extraction solvent with 80 µL, acetone as dispersive solvent with 1.0 mL, without addition of salt.

3.3 Method performance

Under the above optimized separation and extraction conditions, the four PEEs were separated well by CE. Figure 2
shows comparative electropherograms of PEEs standards with (curve a) and without (curve b) DLLME procedure. As seen, the peak height of four PEEs at 100 or 200 µg/L each (curve a) approximated that obtained at 50 or 100 mg/L each (curve b), a 250- or 500-fold higher concentration. Thus, it can be estimated that the EF values due to DLLME were 467, 241, 367 and 362 for HS, BPA, DES and DS, respectively, suggesting the DLLME had high enrichment ability.

Furthermore, the analytical performances of DLLME-CE for PEEs were investigated. Relationship between peak height and concentrations was assessed by using five different concentrations, and good linearity was attained in the range of 2.0–150 µg/L for HS and DS, and 4.0–300 µg/L for BPA and DES, with correlation coefficients (R) over 0.9983. The limits of detection (LODs) determined based on the S/N of 3, were 0.3, 0.6, 0.6 and 0.3 µg/L for HS, BPA, DES and DS, respectively. The values are close to the established maximum residue limits (0.1 µg/L) in nature water, and meet the requirement of trace analysis [26]. As well as, the method can be applied to analyze some wastewater samples especially from feed industry, where the PEEs are possibly present at µg/L or higher. Therefore, the DLLME-CE-DAD method can reach the required levels for the four PEEs in environmental water investigations to some extent.

On the other hand, the RSDs obtained under repeatability conditions, namely the intra-day precisions in terms of migration time were 0.83–0.98%, as listed in Supporting Information Table S1. In a meanwhile, under reproducibility conditions, i.e., the inter-day precisions were in the range of 2.46–3.53%. Thus, the method was proved robust and reliable, and was capable of accurately quantifying PEEs.

In addition, the performances of our developed DLLME-CE were compared with other reported CE and HPLC methods for the PEEs determination. As shown in Table 1, the LODs of our developed method are lower than that of dCPE-CE-DAD [29], dSPE-HPLC-UV [37] and MMIPs-SPME-HPLC-UV [27]. Moreover, the extraction time in DLLME is very short even within a few seconds. However, the above-mentioned three methods require 20 or 40 min for extraction, without equilibrium in most cases. Although higher LODs and longer separation time are obtained compared with the method of DLLME-HPLC-UV [11], our method could simultaneously separate and detect the four PEEs, which has not been reported. More excitingly, the EFs of our method of 241–467 were higher than the method of dCPE-CE-DAD [29], UA-CPE-HPLC-DAD [10] and DLLME-HPLC-UV [35]. Overall, our developed method had obvious superiority based on the low LODs, high EF values and shorter analysis time. In addition, the method was quite simple, fast, cost-saving and eco-friendly. It was potentially suitable for the determination of estrogens in water samples.

### 3.4 Analysis of real water samples

In order to check the practical applicability of the developed method, three kinds of real water samples, i.e., tap water, lake water and seawater were investigated. Figure 3 shows the separation results of lake water samples; Supporting Information Fig. S5 shows the results of other two kinds of water samples. As seen, the endogenous PEEs were not detected in the three water samples (curve a). For the spiked water samples (curve b, c), the four types of PEEs attained complete base-line separation. Obviously, the analysis of the PEEs did not suffer from the interference from water matrices, which is quite probably because of the selective enrichment ability and good cleanup effect of DLLME. Table 2 lists the recoveries of the three water samples by adding three levels of the four PEEs standards. As shown from the table, recoveries ranged from 70.4 to 108.1%, and the RSDs were obtained within 2.1–9.7%. It is also noticeable that the recoveries are satisfactory for seawater samples, in some cases even better than that of tap and lake water. The observations are consistent with the aforementioned salt effect on DLLME. As is well known, seawater usually contains 2.7% NaCl (w/v), and herein, cleaner and lower salt surface seawater samples were used. Thus, the DLLME procedure was applicable for the tested seawater samples, with reasonable results. The developed method was

| PEEs   | Tap water | Lake water | Seawater |
|--------|-----------|------------|----------|
|        | Added (µg/L) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| HS 5   | 79.4       | 7.2        | 70.9     | 5.6        | 86.3    | 5.2        |
| 25     | 94.2       | 5.1        | 72.6     | 4.5        | 77.8    | 7.3        |
| 100    | 99.4       | 5.6        | 102.2    | 7.6        | 97.9    | 6.7        |
| BPA 10 | 77.0       | 7.6        | 77.7     | 7.9        | 89.0    | 7.8        |
| 50     | 99.7       | 2.7        | 90.1     | 7.3        | 108.1   | 7.3        |
| 200    | 103.0      | 5.1        | 97.5     | 7.2        | 104.4   | 5.7        |
| DES 10 | 75.5       | 9.3        | 91.2     | 9.1        | 78.7    | 5.6        |
| 50     | 105.6      | 3.8        | 104.2    | 6.2        | 107.8   | 7.7        |
| 200    | 95.7       | 5.9        | 90.7     | 2.1        | 95.4    | 3.3        |
| DS 5   | 81.7       | 5.4        | 70.4     | 9.7        | 80.8    | 8.9        |
| 25     | 96.2       | 3.9        | 98.2     | 5.7        | 104.1   | 3.5        |
| 100    | 95.3       | 4.0        | 95.1     | 5.8        | 95.1    | 3.9        |

a) Relative standard deviations.
demonstrated practically feasible for the simultaneous separation and determination of trace PEEs in complicated water samples.

4 Concluding remarks

A DLLME-CE method was developed for the separation and determination of four types of PEEs in water samples. By optimizing factors affecting the experiment, high EFs from 241 to 467 and low LODs from 0.3 to 0.6 µg/L were obtained. The method was successfully applied to three kinds of real water samples. The DLLME-CE method with simple UV detector was demonstrated to be a simple, fast, cost-effective and eco-friendly option for simultaneous separation and determination of trace PEEs in complicated aqueous matrices.

On the other hand, more work still needs to be done to further improve the detection sensitivity and promote the advancement of the DLLME-CE method, such as investigating more new types of extraction and dispersive solvents for DLLME, utilizing computation tools for the optimization of influence factors of DLLME, developing various modalities of DLLME, and exploring various CE modes and online enrichment strategies. The dual/multiple CE stacking modes coupled with DLLME modified/derivatized and hyphenated techniques will provide remarkably larger EF and thereby ultrasensitive determination.

We are grateful for financial support from the National Natural Science Foundation of China (No. 51573155, 21477160, 21275158).

The authors have declared no conflict of interest.

5 References

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