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Municipal wastewater treatment plants (WWTPs) act as a major barrier to reduce the release of endocrine disrupting chemicals (EDCs) into the environment. The co-existence of free and conjugated estrogens and the interference from complex matrices often lead to largely variable detected concentrations and sometimes even negative removal efficiencies of typical EDCs in WWTPs. In this study, a highly selective and sensitive method was developed for simultaneous extraction, elution, and detection of 12 EDCs (including 6 conjugated estrogens) in both wastewater and sludge with enhanced sample pretreatment and UPLC-MS/MS. By using the developed method, the behavior of target EDCs in a local anaerobic/anoxic/oxic treatment plant was clarified. This study helps to better understand the behavior and fate of typical EDCs (particularly conjugates) in WWTPs.

Simultaneous detection of endocrine disrupting chemicals including conjugates in municipal wastewater and sludge with enhanced sample pretreatment and UPLC-MS/MS

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ABSTRACT

The co-existence of free and conjugated estrogens and the interference from complex matrices often lead to largely variable detected concentrations and sometimes even negative removal efficiencies of typical endocrine disrupting chemicals (EDCs) in wastewater treatment plants (WWTPs). In this study, a highly selective and sensitive method was developed for simultaneous extraction, elution, and detection of 12 EDCs (i.e., 4 free estrogens, 6 conjugated estrogens, and 2 phenolic compounds) in municipal wastewater and sludge. Sample pretreatment and ultra performance liquid chromatography-tandem mass spectrometry detection were optimized to improve the detection selectivity and sensitivity. Results indicate that the additional purification process was highly effective in reducing the matrix interference, and the limits of quantification reached as low as 0.04–2.2 ng L^{-1} in wastewater and 0.05–4.9 ng g^{-1} in sludge for all target EDCs. The developed method was successfully applied to explore the behavior of target EDCs in a local WWTP. The conjugates occupied a considerable portion (4.3–76.9% in molar ratio) of each related estrogen in the influent. Most of the target EDCs could not be completely removed in WWTPs, thus posing a potential threat to aquatic ecosystems.

Keywords: Endocrine disrupting chemicals; Conjugates; Wastewater treatment plant; Enhanced sample pretreatment; UPLC-MS/MS

1. Introduction

Endocrine disrupting chemicals (EDCs), typically steroidal estrogens including estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2) and endocrine disrupting phenolic compounds including bisphenol A (BPA) and 4-nonylphenol (NP), have drawn much attention in recent years because they may alter the normal hormone functions and physiological status in wildlife and humans.[1] In China, about 790,000 tons of BPA and 41,000 tons of NP were produced and consumed in 2011 (Chinese Statistical Bureau, http://www.stats.gov.cn), and over 10.6 tons of natural estrogens (converted to E2 equivalent) are excreted by humans, livestock, and poultry every year.[2] As a consequence, these compounds have been widely detected in China's surface waters at concentration levels approximately ranging from 1 ng L^{-1} (E3) to 33 μg L^{-1} (NP),[3] which may induce potential ecological risks such as decreasing fertility and causing feminization in fish.[4]

Municipal wastewater treatment plants (WWTPs) act as a major barrier to reduce the release of EDCs into the environment.[5] The occurrence, behavior and fate of EDCs in WWTPs have been extensively investigated.[6,7] However, most of the previous researches only focused on free estrogens and often reported largely variable detected concentrations and sometimes even negative removal efficiencies in WWTPs.[8,9] The reason behind lies in that estrogens are excreted primarily in sulfate and glucuronide conjugated forms in urine and feces, which can readily be transformed to free forms in biological wastewater treatment processes.[10] As a result, an underestimation of removal efficiency will inevitably occur if the conjugated forms

occupy a considerable portion of the total estrogens.[8] This necessitates the detection of conjugated estrogens in WWTPs when investigating the behavior and fate of EDCs.

A number of analytical methods have been developed to identify and quantify typical EDCs in municipal wastewater and sludge;[11] however, few methods have achieved simultaneous extraction and detection of both free and conjugated estrogens. Conjugated estrogens cannot be directly analyzed by gas chromatography and mass spectrometry (GC-MS) or bioassays but need to undergo an enzymatic or acidic hydrolysis to free forms prior to analysis.[12,13] This renders the detection methods rather complicated and cannot differentiate the original forms of conjugated estrogens.

Recently, ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) has become a major means to detect free and conjugated estrogens in wastewater, sludge and other environmental matrices;[14–16] however, most of the earlier developed methods had to detect free and conjugated estrogens separately,[15] or were not able to extract glucuronide conjugates simultaneously.[14] In addition, LC is more easily interfered by sample matrix than GC, which considerably reduces the detection sensitivity.[17] Although using tandem mass can reduce the possibility of false positives and thus increase the detection selectivity, co-eluted impurities from sample pretreatment process may significantly suppress the ionization of target analytes.[18] Hence, much effort has been put to reduce matrix interference through improving sample pretreatment (e.g., applying extra rinsing steps and/or purification cartridges) to further clean up samples.[15,19] However, these approaches are usually designated for

a single group of EDCs (e.g., conjugates).

Therefore, this study aimed to develop a highly selective and sensitive method for simultaneous detection of 12 EDCs (including 4 free estrogens, 6 conjugated estrogens, BPA, and NP) in municipal wastewater and sludge. Both sample pretreatment and UPLC-MS/MS detection were optimized to simultaneously extract, elute, and detect all target EDCs with minimized matrix interference, which significantly improved the detection selectivity and sensitivity. The calibration linearity, method recovery, and limit of quantification (LOQ) were all assessed for method validation. Afterwards, the developed method was applied to explore the behavior of target EDCs (particularly conjugates) in a WWTP located in Beijing, China.

2. Materials and methods

2.1. Chemicals

E1, E2, E3, EE2, and BPA standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany; purity > 99.0%), and 4-NP (> 95.0%, CAS 25154-52-3) was from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Sodium salts of 6 conjugated estrogens (> 95% purity), including estrone 3-β-D-glucuronide (E1-3G), β-estradiol 3-β-D-glucuronide (E2-3G), β-estradiol 17-β-D-glucuronide (E2-17G), estrone 3-sulfate (E1-3S), β-estradiol 3-sulfate (E2-3S), and estriol 3-sulfate (E3-3S) were purchased from Sigma Aldrich China (Shanghai, China). Three surrogate standards, including E2-d_2 (≥ 98%), BPA-d_{16} (≥ 98%), and E2-3S-d_4 (50% Tris), were supplied by CDN Isotopes (Quebec, Canada).

High performance liquid chromatography (HPLC) grade methanol (MeOH), methyl *t*-butyl ether (MTBE), acetonitrile (ACN), dichloromethane (DCM), and acetone (ACE) were purchased from Fisher Scientific (Geel, Belgium). Milli-Q (MQ) water was produced with a Millipore purification system (Advantage A10, Millipore, Bedford, US). HPLC grade ammonia (25% NH_4OH in water, by weight) was purchased from Sigma Aldrich China. The stock solutions of target EDCs and surrogate standards were individually prepared by dissolving each compound in MeOH at a concentration of 1000 mg L^{-1} and stored at –20°C in refrigerator. Na_2EDTA-McIlvaine buffer (MB) was prepared by dissolving 21.00 g citric acid monohydrate, 17.75 g Na_2HPO_4, and 60.50 g $Na_2EDTA{\cdot}2H_2O$ in 1.625 L of deionized water, with the pH adjusted to 4.00 ± 0.05.[20]

2.2. Sample collection

Wastewater and sludge samples were collected from a municipal WWTP located in Beijing, China, which primarily adopts an anaerobic/anoxic/oxic (A/A/O) biological treatment process. The sampling points are illustrated in Fig. S1, except digested sludge samples which were collected from a sludge digestion tank. The samples of excess sludge and digested sludge were grabbed three times on a sampling day (i.e., in the morning, noon, and evening) and mixed together. All other samples were acquired as the flow-proportional (24 h) mixture by using electronic auto-samplers (SD900, Hach, Loveland, CO, US). The samples were stored in 4 L amber glass bottles, in which 1% MeOH (v/v) was pre-added to inhibit microbial activity, and transported immediately to laboratory.

2.3. Sample pretreatment

The sample pretreatment procedures were optimized stepwise, as detailed in Fig. 1. Wastewater samples, including the influent, mixed liquor from each treatment unit of the A/A/O process, and effluent, were centrifuged at 6000 revolutions per minute (rpm) for 10 min. The supernatant (400 mL each) was adjusted to pH 3.0 with 40% H_2SO_4 (v/v), filtered through GF/F glass microfiber filters (0.7 μm, Whatman, UK), spiked with the surrogate standard solutions (i.e., 50 ng L^{-1} E2-3S-d_4, 100 ng L^{-1} E2-d_2, and 200 ng L^{-1} BPA-d_{16}), stored at 4 °C in refrigerator, and subjected to solid-phase extraction (SPE) within 24 h.

Fig. 1

Sludge samples (i.e., excess and digested) were centrifuged at 6000 rpm for 10 min to collect solid particles. The solid particles, as well as the mixed liquor suspended solids from each treatment unit of the A/A/O process, were freeze-dried under vacuum (FD-1-50, Boyikang, China), homogenized using a mortar and pestle, and sieved to obtain the desired particles (diameter ≤ 0.5 mm). One gram of the sieved particles was weighted and placed into a 10 mL glass centrifuge tube, spiked with the surrogate standard solutions (i.e., 20 ng g^{-1} E2-3S-d_4, 40 ng g^{-1} E2-d_2, and 80 ng g^{-1} BPA-d_{16}), vortexed vigorously to mix the surrogate standards with the sludge particles, and subjected to ultrasonic solvent extraction (USE). Each sample was added with a test extraction solvent (5 mL, as detailed in Section 3.2), vortexed vigorously, ultrasonicated for 10 min, and centrifuged at 5000 rpm for 8 min to collect the supernatant. The USE was continuously performed for three times, and the

supernatants were mixed together, diluted to a total volume of 400 mL with MQ water, filtrated through GF/F glass microfiber filters, adjusted to pH 3.0 with 40% H_2SO_4 (v/v), stored at 4 °C in refrigerator, and subjected to SPE within 24 h

An Oasis HLB cartridge (500 mg/6 mL, Waters), after being activated by 5 mL each of MTBE, MeOH, and MQ water, was applied to concentrate the above-pretreated wastewater or sludge sample at a flow rate of 3–5 mL min^{-1}. Thereafter, the cartridge was sequentially rinsed with 5 mL each of 10% MeOH aqueous solution, MQ water (pH 3.0), 2:10:88 NH_4OH/MeOH/MQ water (v/v/v, pH 11.0), and 30% MeOH aqueous solution to eliminate impurities from sample matrix, and then was dried under vacuum.

Before elution, a Sep-pak C18 cartridge (500 mg/6 mL, Waters), pre-activated by 5 mL of the test eluent, was connected below the dried HLB cartridge to further clean the impurities. For sludge samples, 0.5 g Al_2O_3 and 1.0 g Na_2SO_4 were sequentially packed into its headspace to retain the co-eluted impurities and dehydrate the eluate from the HLB cartridge, respectively. Nie et al.[21] reported a post-SPE cleanup procedure by using a laboratory-made glass column, which was filled with anhydrous Na_2SO_4, neutral Al_2O_3, and silica gel from top to bottom, to reduce the co-eluted impurities. Afterwards, the HLB cartridge was eluted with 2 × 4 mL of a selected eluent. The eluates were collected into a 10 mL conical-bottomed glass tube, placed in a water bath at 35 °C, and dried under a gentle stream of N_2. The dried residue was immediately reconstituted with 200 μL of 9:1 MQ water/MeOH (v/v) for UPLC-MS/MS detection.

2.4. UPLC-MS/MS analysis

An Agilent 1290 UPLC system, equipped with a BEH-C18 column (100 mm × 2.1 mm, 1.7 μm, Waters) at a constant temperature of 30 °C, was used for separation of target analytes. The injection volume was 10 μL. The mobile phases consisted of MQ water (A) and a mixed solvent of 1:1:8 MeOH/MQ water/ACN (v/v/v) (B), with each containing 7.5 mmol L^{-1} NH_4OH to improve the ionization efficiency in the tandem MS system. The mobile phases had a total flow rate of 0.2 mL min^{-1} and a gradient elution program (time in min, % mobile phase B) as follows: (0, 10), (5, 40), (5.1, 50), (8, 50), (13, 70), (14, 100), (17, 100), (17.1, 10), and (21, 10). All 12 target EDCs could be eluted within 18 min.

An Agilent 6420 triple quadrupole MS, operated in the negative electrospray ionization (ESI(–)) mode with a capillary voltage of 3.5 kV, was used to detect target analytes. Multiple reaction monitoring (MRM) mode was adopted for data acquisition. The operational parameters of the tandem MS, including fragmentor voltage, and product ions and associated cone energies, were optimized for each analyte, as listed in Table S1.

2.5. Method validation

The method was validated by measuring the calibration linearity, inter- and intra-day precisions, method recoveries, and LOQs. For each analyte, a 10-point calibration curve was established with concentrations ranging from 5 to 5000 ng L^{-1} for NP and 0.5 to 500 ng L^{-1} for all other analytes. The intra-day precision was evaluated by analyzing the target EDCs at three concentration levels (i.e., 5, 50 and

500 ng L^{-1}, spiked in MQ water) every four hours for three times under the routine use of the developed method, and the inter-day precision was assessed by repeating the intra-day analysis every two days for three times.

To determine the method recoveries of target EDCs in wastewater, 200 μL of a mixed standard solution prepared in MeOH (containing 50 μg L^{-1} of each conjugate, 100 μg L^{-1} of each free estrogen and BPA, and 1000 μg L^{-1} of NP) was spiked into a 400 mL wastewater sample. To determine the method recoveries in sludge, certain volumes (200 and 600 μL) of the above mixed standard solution were spiked into 1.0 g of the freeze-dried and sieved aerobic sludge (as reference matrix) to achieve the low and high concentration levels, respectively. The spiked wastewater and sludge samples, together with their raw samples (to measure the background concentrations of target EDCs), were pretreated (Fig. 1) and analyzed by UPLC-MS/MS. The method recovery of each analyte was calculated as follows:

$$Recovery(\%) = \frac{C_{spi} - C_{raw}}{C_{std}} \times 100 \quad (1)$$

where C_{spi}, C_{raw} and C_{std} are the concentrations of a target analyte in the spiked sample, raw sample and standard solution, respectively. The LOQ in wastewater or sludge was determined separately by spiking a mixed standard solution into the SPE eluate, which was then serially diluted to find an analyte concentration that provided a signal to noise ratio of 10:1.[22]

3. Results and discussion

3.1. Optimization of UPLC-MS/MS operational parameters

The operational parameters of MS/MS were optimized under the ESI(−) mode by directly injecting 1000 μg L^{-1} standard solution of each analyte into the tandem MS. Because of the instable ion fragmentations in the collision cell when the steroidal rings were cleaved to form product ions, a "broad" selection mode (allowing ions with ± 0.3 m/z shift to enter the MS2 detector rather than the instrument default "unit" selection mode with ± 0.1 m/z shift) was employed to identify the product ions of four free estrogens (i.e., E1, E2, E3, EE2). For example, the 145.18 m/z ion was formed as a major product ion from the fragmentation of E2 (Fig. S2a). Its signal peak could alter by 0.2 m/z in the MS2 scan mode among different injections (e.g., between the 1^{st} and 2^{nd} injections), and alter by 0.1 m/z among different retention times in the same injection (e.g., between the retention times of 3.642 and 3.706 min in the 2^{nd} injection) (Fig. S2b). Hence, the default "unit" selection mode may considerably reduce the response of product ions. By using the "broad" selection mode, the detection sensitivity of product ions could be enhanced by at least 4 folds.

Various UPLC conditions were tested to improve the resolution and sensitivity of target EDCs. Relatively smooth alteration of mobile phase ratios was employed in the first 13 min to achieve an effective separation of 11 analytes, and then the ratio of mobile phase B was rapidly raised to elute NP. The total-ion MRM chromatogram of 12 EDCs is shown in Fig. 2. Except the three deuterated standards that were co-eluted with their non-deuterated counterparts (i.e., E2-3S, BPA, and E2), all other EDCs were well separated.

Fig. 2

3.2. USE optimization

It was reported that 1:1 MeOH/ACE (v/v) could efficiently extract free estrogens and phenolic EDCs from sludge in the USE process.[21] In addition, an acidic buffer (e.g., MB or citric acid (CA)) in combination with an organic solvent has also been proved efficient for extracting free estrogens from soils.[23] To extract conjugated estrogens from sludge, a mixed solvent of MeOH/DCM or MeOH/ACE was used in an accelerated solvent extraction (ASE) process;[14,24] however, the suitable extraction solvent for conjugates in the USE process has never been reported. In the present study, MeOH, MB, CA, and their pair-wise combinations (1:1 of MeOH/MB, MeOH/CA, and MeOH/ACE) were examined individually for simultaneous extraction of all 12 target EDCs from sludge.

The extraction efficiency of each target analyte is shown in Fig. 3. MeOH showed an acceptable extraction efficiency for most free estrogens (i.e., E1, E2, EE2) and phenolic compounds, but a low efficiency (< 50%) for E3 (relatively hydrophilic, log K_{ow} = 2.81), E2-3G, E2-17G, and E3-3S. CA and MB were effective in extracting the conjugates but failed to extract the phenolic compounds (< 40%). Although 1:1 MeOH/ACE was reported to be effective in extracting both free and conjugated estrogens in the ASE process,[14] it was ineffective in extracting most conjugates in the USE process. Both 1:1 MeOH/CA and 1:1 MeOH/MB could simultaneously extract most of the target EDCs with an acceptable efficiency (> 50%). Because 1:1 MeOH/MB exhibited a better extraction efficiency for E1-3S, EE2 and NP, it was selected as the optimal extraction solvent in the USE process. Using this solvent, all

target EDCs could be extracted with an efficiency ranging from 57.1% to 109.4%.

Fig. 3

3.3. SPE optimization

HLB cartridge is commonly used in the SPE process because it contains both hydrophobic and hydrophilic units to retain a wide range of compounds. However, this advantage also makes it less selective in a complex matrix. In this regard, a four-step sequential rinsing procedure was developed to reduce the matrix interference (Fig. 1). The first three solvents with an increasing polarity (i.e., 10% MeOH, MQ water (pH 3.0), and 2:10:88 NH_4OH/MeOH/MQ water (pH 11.0)) were used to rinse off the retained hydrophilic matrix compounds, while the fourth solvent (i.e., 30% MeOH) was used to rinse off the retained hydrophobic compounds.

After connection of a Sep-pak C18 cartridge to the rinsed and vacuum dried HLB cartridge, sequential elutions with 3:2 DCM/ACE (v/v), MeOH, and MeOH containing 5% NH_4OH (by weight) were performed to determine the optimal eluent composition. The cumulative recoveries of each target analyte by different eluents are presented in Fig. 4. Results show that the first elution with DCM/ACE was effective for the free estrogens and phenolic compounds (recovery = 82–110%), but ineffective for the conjugates (recovery = 0–4%). The second elution with MeOH could elute most of the conjugates (recovery = 68–83%) and 16.8% of NP. However, the retained conjugates were resistant to further elution with a more polar eluent (i.e., MeOH containing 5% NH_4OH), indicating a further increase in the eluent polarity was not necessary. Hence, a mixture of DCM/ACE and MeOH (3:2:5, v/v/v) was selected as

the optimal eluent in the SPE process.

Fig. 4

To evaluate the efficacy of the additional purification process (i.e., four-step sequential rinsing plus Sep-pak C18 cleanup), four different matrices, including the influent, effluent, aerobic sludge, and digested sludge collected from a local WWTP, were utilized to compare the matrix effects (ME%) between the purified and unpurified samples. The purified samples were spiked with 200 μL of a mixed standard solution (100 μg L^{-1} of each target analyte, prepared in MQ water) and subjected to the additional purification process (Fig. 1), whereas the unpurified samples were spiked identically but did not undergo the additional purification process. The ME% value was calculated as follows:

$$ME(\%) = \frac{A_{spi} - A_{raw}}{A_{std}} \quad (2)$$

where A_{spi}, A_{raw} and A_{std} are the signal responses of a target analyte in the spiked sample, raw sample and standard solution, respectively. To reflect the real matrix interference (i.e., signal suppression or enhancement), surrogate standard correction was not adopted for the ME% calculation.

Fig. 5 shows that the additional purification process could obviously reduce the matrix interference for both wastewater and sludge samples. The ME% values of almost all target EDCs (except NP) in the unpurified samples were considerably lower than those in the purified samples, implying that both wastewater and sludge matrices severely suppressed the signal responses. On the contrary, the signal response of NP was significantly enhanced by the unpurified sludge matrix (ME% = 129–133). The

total-ion MRM chromatograms of all target EDCs in the standard solution, unpurified sludge sample, and purified sludge sample are comparatively illustrated in Fig. S3, which demonstrates that the additional purification process was highly effective in reducing the matrix interference.

Fig. 5

3.4. Method validation

The calibration curve of each target analyte exhibited a good linearity ($R^2 > 0.99$) over a broad concentration range (Table S1). The intra- and inter-day precisions are expressed by the recovery and relative standard deviation (RSD) values of repeated analyses. Table S2 shows that in the intra-day precision tests, the recoveries of target analytes ranged from 86.3 to 110.7% for all three concentration levels, with RSD values below 15%. Meanwhile, in the inter-day precision tests, the recoveries ranged from 84.3 to 107.7%, with RSD values below 19%. Both intra- and inter-day precisions confirmed the good repeatability of the developed method.

As shown in Table 1, the majority of recovery efficiencies were within the range of 70–120% and all the RSD values were below 20% (as recommended by the U.S. EPA) in different test matrices, which ensures the accuracy and robustness of the developed method. Only a few recovery efficiencies of E3 (130.9%), E1-3G (48.5–66.5%), E2-17G (68.5% and 68.2%), and E1-3S (63.5%) in wastewater and sludge exceeded the recommended range to some extent, but these recoveries were quite stable as reflected by their RSD values (i.e., 2.9–13.4%). On the whole, the recovery efficiency deviated farther from the ideal value (100%) as the sample matrix

became more complex (i.e., from MQ water to effluent, influent, and sludge).

Table 1

In MQ water, the LOQs of 12 target EDCs ranged from 0.03 to 1.1 ng L^{-1}. In the influent and effluent, the LOQs were in the ranges of 0.07–2.2 and 0.04 –1.4 ng L^{-1}, respectively. Several earlier developed methods for detection of free or conjugated estrogens reported the LOQs of 0.5–30,[10] 0.4–3.0,[15] and 15–75 ng L^{-1} [25] in the influent and effluent of WWTPs. In the sludge, the LOQs of free estrogens, conjugated estrogens, and NP were 0.5–3.4, 0.05–1.5, and 4.9 ng g^{-1}, respectively; which are considerably lower than the previously reported values (i.e., 1.2–10.0, 0.3–5.0, and 188.1 ng g^{-1}, correspondingly).[14,21,26] This result demonstrates that the additional purification process could significantly improve the detection sensitivity, which makes the developed method well applicable to the detection of target EDCs in both wastewater and sludge of WWTPs.

3.5. Method application

The developed method was applied to determine the concentrations of target EDCs along the A/A/O treatment process in a local WWTP. The major characteristics of the influent and effluent of this WWTP are shown in Table S3. All target EDCs were detected in the influent with a maximum concentration of 333 ng L^{-1} (E1) for free estrogens, 39.1 ng L^{-1} (E3-3S) for conjugated estrogens, and 2319 ng L^{-1} (NP) for phenolic compounds (Table 2). It should be noticed that the conjugates (sulfate plus glucuronide) occupied 4.3%, 76.9% and 25.5% (molar ratio) of the total E1, E2 and E3, respectively, implying that a considerable portion of estrogens entered the

WWTP in conjugated forms. Liu et al.[27] also reported similar conjugate concentrations in the influent of a municipal WWTP located in Osaka, Japan, where the concentrations of E1-3S/E1-3G and E3-3S were measured to range from 3.2 to 21.9 ng L^{-1}. However, the conjugates in their study occupied 33.2% and 100.0% (molar ratio) of the total E1 and E3, respectively. The different molar ratios could partly arise from the different sampling seasons (i.e., April in this study and January in their study) because the enzymatic hydrolysis of conjugated estrogens in sewer pipes is promoted at an increased wastewater temperature.[28]

Table 2

After entering the anaerobic zone, E1, EE2, BPA, and NP were removed quite effectively in wastewater but were found to be abundant in sludge, probably because of their relatively high LogK_{ow} values (> 3.0) which facilitated their absorption onto sludge particles.[7] E2 exhibited an increased concentration in wastewater, which probably arose from the effective transformation of E2-3G and E2-17G via enzymes,[10] as evidenced by their negligible concentrations in both wastewater and sludge. E2-3S was significantly distributed in sludge, whose release from sludge might explain its increased concentration in wastewater. The concentrations of E3 and E3-3S in wastewater maintained nearly constant after entering the anaerobic zone.

In the anoxic and oxic zones, the concentrations of free estrogens and phenolic compounds continuously decreased in wastewater and sludge, probably because of the co-metabolic degradation by nitrifying bacteria.[5] The glucuronide conjugates were rarely detected, while the sulfate conjugates appeared to persist in wastewater and

sludge although some decay was observed for E1-3S and E3-3S. It was reported that more enzymes are usually present for glucuronide conjugates than for sulfate conjugates in biological treatment processes.[10]

In the effluent and excess sludge, only EE2 and the glucuronide conjugates were completely removed. E1, E2, E3, and their sulfate conjugates were still detectable with concentration ranges of 1.8–14.8 ng L^{-1} and 0.4–16.1 ng g^{-1} in the effluent and excess sludge, respectively. A considerable amount of BPA and particularly NP would be released into the environment through effluent discharge and sludge disposal. It is seen that most of the target EDCs could not be completely removed by the wastewater treatment processes, thus posing a potential threat to aquatic ecosystems. In addition, the results demonstrate that the removal efficiency of estrogens can be significantly underestimated if the conjugated forms are ignored. Taking E2 as example, its removal efficiency was 58.0% if only considering the free form, but could reach 83.4% if considering both the free and conjugated forms.

4. Conclusions

This study developed a highly selective and sensitive method for simultaneous extraction, elution, and detection of 12 EDCs (i.e., 4 free estrogens, 6 conjugated estrogens, and 2 phenolic compounds) in both wastewater and sludge of WWTPs. Based on the experimental results, the following conclusions can be drawn:

- 1:1 MeOH/MB and 3:2:5 DCM/ACE/MeOH (by volume) were the optimal extraction solvents in the USE and SPE processes, respectively. The additional purification process (i.e., four-step sequential rinsing plus Sep-Pak C18 cartridge

cleanup) was highly effective in reducing the matrix interference.

- The LOQs of 12 target EDCs were in the ranges of 0.04–2.2 ng L^{-1} and 0.05–4.9 ng g^{-1} in the wastewater (i.e., influent and effluent) and sludge, respectively. The majority of recovery efficiencies were within the range of 70–120% and all RSD values were below 20%, which ensures the detection accuracy and precision.
- The developed method was applied to explore the behavior of target EDCs in a local WWTP. All 12 target EDCs were detected in the influent, where the conjugates occupied a considerable portion (4.3–76.9% in molar ratio) of each related estrogen (i.e., E1, E2, and E3). Only EE2 and the glucuronide conjugates were completely removed in the effluent and excess sludge, while other studied EDCs were partially removed by the wastewater treatment processes.
- Further research should be conducted to clarify the transformation mechanism of conjugated estrogens in WWTPs and assess the potential ecological risk of residual EDCs in the effluent and excess sludge.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version

at.

References

1. D. P. Grover, J. Balaam, S. Pacitto, J. W. Readman, S. White and J. L. Zhou, *Chemosphere*, 2011, **84**, 1512–1520.
2. W. An and J. Y. Hu, *Front. Ecol. Environ.*, 2006, **4**, 378–386.
3. X. Z. Peng, Y. J. Yu, C. M. Tang, J. H. Tan, Q. X. Huang and Z. D. Wang, *Sci. Total Environ.*, 2008, **397**, 158–166.
4. C. G. Campbell, S. E. Borglin, F. B. Green, A. Grayson, E. Wozei and W. T. Stringfellow, *Chemosphere*, 2006, **65**, 1265–1280.
5. Z. H. Liu, Y. Kanjo and S. Mizutani, *Sci. Total Environ.*, 2009, **407**, 731–748.
6. H. D. Zhou, X. Huang, X. L. Wang, X. H. Zhi, C. D. Yang, X. H. Wen, Q. H. Wang, H. Tsuno and H. Tanaka, *Environ. Monit. Assess.*, 2010, **161**, 107–121.
7. Z. M. Qiang, H. Y. Dong, B. Zhu, J. H. Qu and Y. F. Nie, *Chemosphere*, 2013, **92**, 986–992.
8. Z. H. Liu, G. N. Lu, H. Yin, Z. Dand, and B. Rittmann, *Environ. Sci. Technol.*, 2015, **49**, 5288–5300.
9. H. Hamid and C. Eskicioglu, *Water Res.*, 2012, **46**, 5813–5833.
10. G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Mancini, R. Mastropasqua, M. Nazzari and R. Samperi, *Sci. Total Environ.*, 2003, **302**, 199–209.
11. A. D. LaFleur and K. A. Schug, *Anal. Chim. Acta.*, 2011, **696**, 6–26.
12. A. C. Belfroid, A. Van der Horst, A. D. Vethaak, A.J. Schafer, G. B. J. Rijs, J. Wegener and W. P. Cofino, *Sci. Total Environ.*, 1999, **225**, 101–108.

13. Z. H. Liu, Y. Kanjo and S. Mizutani, *J. Environ. Monitor.*, 2010, **12**, 1594–1600.

14. A. Nieto, F. Borrull, E. Pocurull and R. M. Marce, *J. Chromatogr. A*, 2008, **1213**, 224–230.

15. V. Kumar, N. Nakada, M. Yasojima, N. Yamashita, A. C. Johnson and H. Tanaka, Chemosphere, 2009, **77**, 1440–1446.

16. S. Dutta, S. Inamdar, J. Tso, D. S. Aga, J. T. Sims, *J. Environ. Qual.*, 2010, **39**, 1688–1698.

17. T. Reemtsma, *J. Chromatogr. A*, 2003, **1000**, 477–501.

18. C. Miege, P. Bados, C. Brosse and M. Coquery, *Trac-Trend Anal. Chem.*, 2009, **28**, 237–244.

19. R. P. S. Suri, T. S. Singh and R. F. Chimchirian, *Environ. Monit. Assess.*, 2012, **184**, 1657–1669.

20. X. Pan, Z. M. Qiang, W. W. Ben and M. X. Chen, *Chemosphere*, 2011, **84**, 695–700.

21. Y. F. Nie, Z. M. Qiang, H. Q. Zhang, C and Adams, *J. Chromatogr. A*, 2009, **1216**, 7071–7080.

22. J. Tso and D. S. Aga, *J. Chromatogr. A*, 2010, **1217**, 4784–4795.

23. F. Chen, G. G. Ying, L. X. Kong, L. Wang, J. L. Zhao, L. J. Zhou and L. J. Zhang, *Environ. Pollut.*, 2011, **159**, 1490–1498.

24. M. P. Fernandez, T. N. Noguerol, S. Lacorte, I. Buchanan and B. Pina, *Anal. Bioanal. Chem.*, 2009, **393**, 957–968.

25. M. Pedrouzo, F. Borrull, E. Pocurull and R. M. Marce, *Talanta*, 2009, **78**,

1327–1331.

26. C. E. Bevacqua, C. P. Rice, A. Torrents and M. Ramirez, *Sci. Total Environ.*, 2011, **409**, 2120–2126.

27. Z. H. Liu, T. Hashimoto, Y. Okumura, Y. Kanjo and S. Mizutani, *Clean-Soil Air Water*, 2010, **38**, 181–188.

28. R. L. Gomes, M. D. Scrimshaw and J. N. Lester, *Environ. Sci. Technol.*, 2009, **43**, 3612–3618.

Figure Captions

Fig. 1 Schematic diagram of the optimized sample pretreatment procedures.

Fig. 2 Total-ion MRM chromatogram of 12 target EDCs and 3 surrogate standards.

Fig. 3 Extraction efficiencies of different solvents for 12 target EDCs in the ultrasonic solvent extraction process (ACE: acetone; CA: citric acid; MB: McIlvaine buffer; MeOH: methanol).

Fig. 4 Cumulative recoveries of sequential elutions with different solvents for 12 target EDCs in the solid-phase extraction process (ACE: acetone; DCM: dichloromethane; MeOH: methanol).

Fig. 5 Matrix effects of 12 target EDCs detected with/without additional purification in spiked wastewater and sludge samples (P: purified; UP: unpurified).

Table 1

Recoveries and limits of quantification (LOQs) for 12 target EDCs in MQ water, wastewater, and sludge.

Analyte	MQ water		Influent		Effluent		Sludge		
	Recovery (RSD[a]) %	LOQ (ng L^{-1})	Recovery (RSD) %	LOQ (ng L^{-1})	Recovery (RSD) %	LOQ (ng L^{-1})	LL[b] Recovery (RSD) %	HL[b] Recovery (RSD) %	LOQ (ng g^{-1})
E1	106.7 (1.8)	0.2	112.9 (13.8)	0.1	103.9 (3.9)	0.1	85.8 (5.0)	106.2 (10.6)	0.5
E2	103.5 (2.5)	0.6	97.8 (3.9)	0.6	101.7 (4.2)	0.5	94.4 (9.4)	96.2 (2.4)	1.9
E3	98.4 (3.6)	0.5	130.9 (4.5)	1.5	97.3 (9.2)	0.9	75.4 (5.5)	93.4 (2.0)	1.0
EE2	97.8 (2.6)	0.9	80.9 (4.6)	0.8	122.4 (0.7)	0.8	94.4 (10.4)	83.5 (8.3)	3.4
BPA	111.8 (3.7)	0.5	107.1 (18.7)	1.0	103.4 (1.5)	0.7	92.6 (17.0)	93.7 (7.7)	1.2
NP	84.5 (14.0)	1.0	78.5 (1.5)	2.2	80.6 (11.9)	0.7	101.1 (8.2)	77.6 (9.4)	4.9
E1-3G	99.1 (8.0)	0.3	48.5 (3.8)	1.0	61.2 (3.0)	0.6	66.5 (5.1)	59.7 (7.9)	0.4
E2-3G	92.1 (5.8)	0.3	92.3 (7.3)	0.7	86.2 (4.4)	0.5	71.4 (13.7)	62.8 (5.5)	0.7
E2-17G	88.7 (5.3)	1.1	92.8 (7.4)	1.7	81.6 (3.7)	1.4	68.5 (9.9)	68.2 (2.9)	1.5
E1-3S	86.6 (1.5)	0.03	76.9 (2.9)	0.07	89.4 (2.5)	0.05	63.5 (13.4)	83.3 (6.3)	0.07
E2-3S	99.7 (0.6)	0.03	83.2 (3.3)	0.07	98.5 (1.5)	0.04	76.3 (7.0)	79.9 (9.5)	0.05
E3-3S	98.9 (1.2)	0.04	114.9 (2.3)	0.08	84.8 (5.3)	0.07	92.1 (9.9)	90.3 (9.3)	0.07

[a] Relative standard deviation, $n = 3$.

[b] LL: low concentration level (10 ng g^{-1} of each conjugated estrogen, 20 ng g^{-1} of each free estrogen and BPA, and 200 ng g^{-1} of NP); HL: high concentration level (30 ng g^{-1} of each conjugated estrogen, 60 ng g^{-1} of each free estrogen and BPA, and 600 ng g^{-1} of NP).

Table 2

Concentrations of 12 target EDCs in wastewater and sludge along the biological treatment process (A/A/O) in a local WWTP (April, 2013).

Analyte	Influent	Anaerobic zone		Anoxic zone		Oxic zone		Effluent	Excess sludge
	($ng\ L^{-1}$)	Wastewater ($ng\ L^{-1}$)	Sludge ($ng\ g^{-1}$)	Wastewater ($ng\ L^{-1}$)	Sludge ($ng\ g^{-1}$)	Wastewater ($ng\ L^{-1}$)	Sludge ($ng\ g^{-1}$)	($ng\ L^{-1}$)	($ng\ g^{-1}$)
E1	333 (4)[a]	159 (5)	53.6 (6.5)	54.0 (1.5)	38.7 (0.8)	16.3 (0.3)	17.9 (1.0)	14.8 (3.4)	16.1 (4.4)
E2	5.0 (0.7)	14.5 (1.0)	8.3 (0.7)	3.3 (0.5)	12.3 (1.4)	1.8 (0.4)	6.8 (0.1)	2.1 (0.3)	4.3 (0.5)
E3	89.0 (1.7)	84.8 (1.1)	42.6 (6.8)	37.0 (2.7)	25.0 (3.3)	6.7 (0.9)	3.9 (0.3)	2.1 (0.4)	1.9 (0.1)
EE2	28.6 (6.5)	<LOQ[b]	139 (3)	<LOQ	105 (16)	<LOQ	1.2 (0.7)	<LOQ	<LOQ
BPA	191 (10)	148 (7)	371 (58)	87.0 (10.8)	262 (39)	86.5 (8.9)	149 (10)	13.0 (0.6)	92.3 (5.1)
NP	2319 (17)	1496 (62)	13327 (3311)	917 (117)	6547 (16)	774 (56)	5983 (1437)	676 (11)	3579 (672)
E1-3G	3.6 (0.6)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
E2-3G	13.3 (1.2)	1.2 (0.2)	<LOQ	1.0 (0.3)	<LOQ	0.9 (0.3)	<LOQ	<LOQ	<LOQ
E2-17G	10.5 (2.3)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
E1-3S	16.5 (0.9)	9.0 (0.4)	<LOQ	3.4 (0.2)	<LOQ	1.6 (0.1)	<LOQ	1.9 (0.4)	0.7 (0.1)
E2-3S	2.7 (0.1)	9.2 (0.3)	10.2 (0.3)	1.8 (0.2)	13.3 (1.1)	1.3 (0.1)	17.3 (0.9)	1.8 (0.5)	11.0 (0.7)
E3-3S	39.1 (3.4)	39.0 (1.4)	1.3 (0.1)	8.5 (0.5)	0.7 (0.1)	5.0 (2.4)	0.7 (0.1)	2.0 (0.2)	0.4 (0.1)

[a] Mean concentration (SD), $n = 3$.

[b] The LOQs of target EDCs in wastewater and sludge are provided in Table 1.

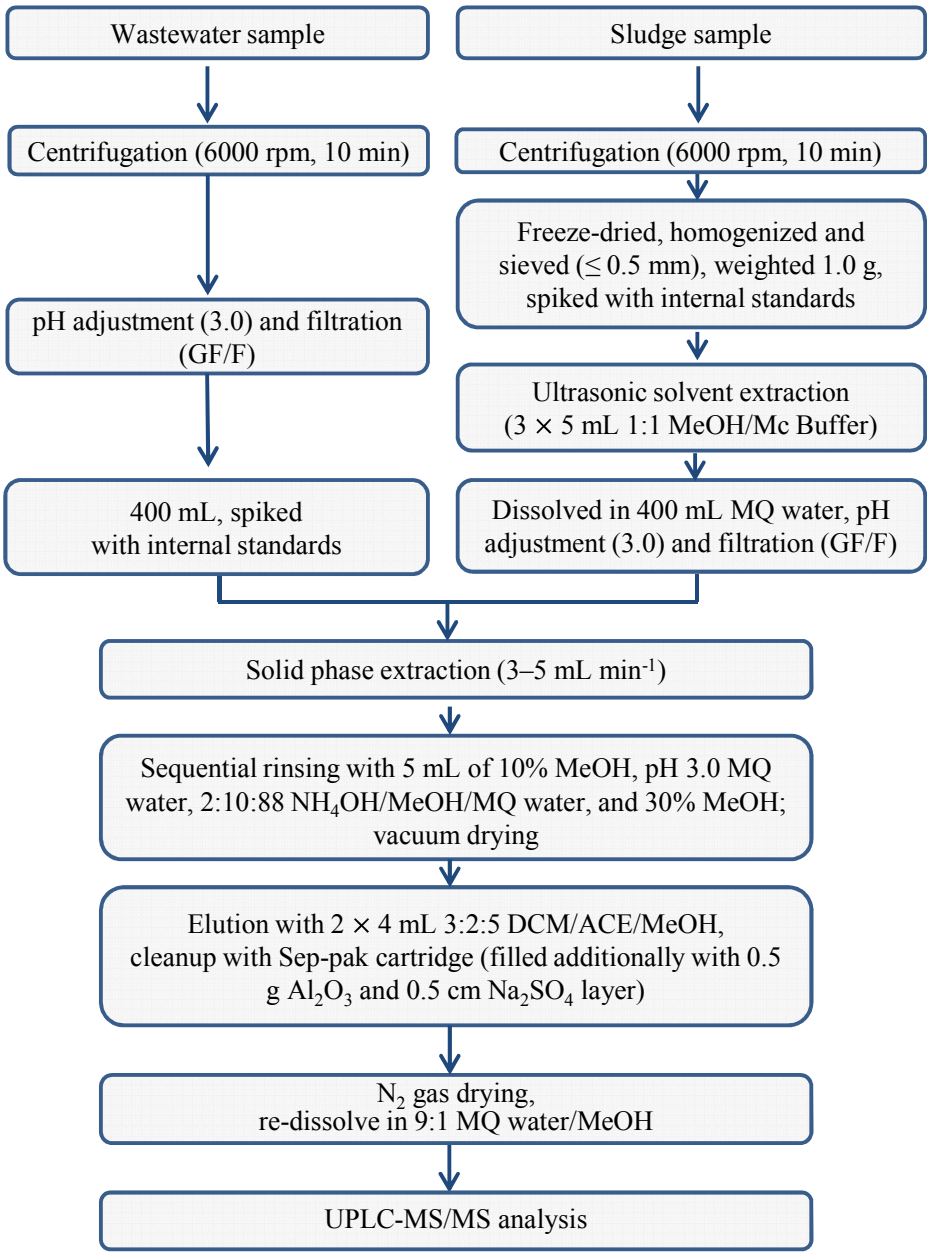


Fig. 1. Schematic diagram of the optimized sample pretreatment procedures.

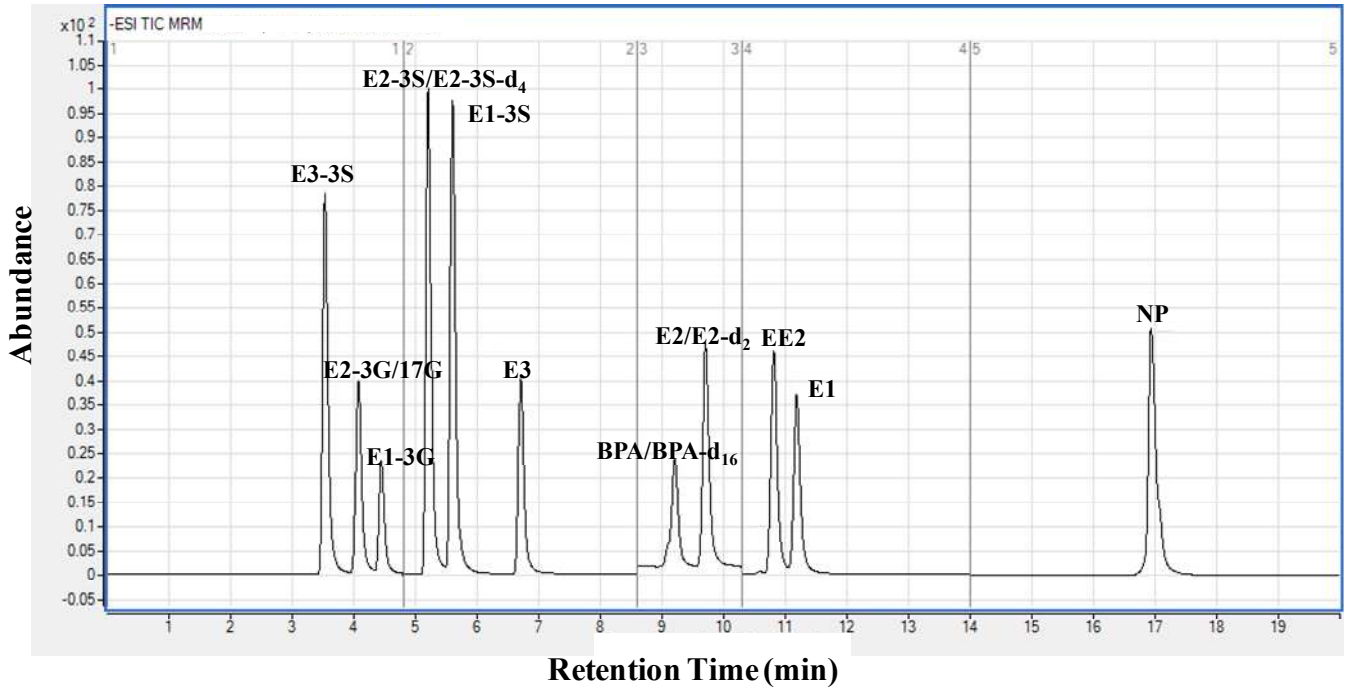


Fig. 2. Total-ion MRM chromatogram of 12 target EDCs and 3 surrogate standards.

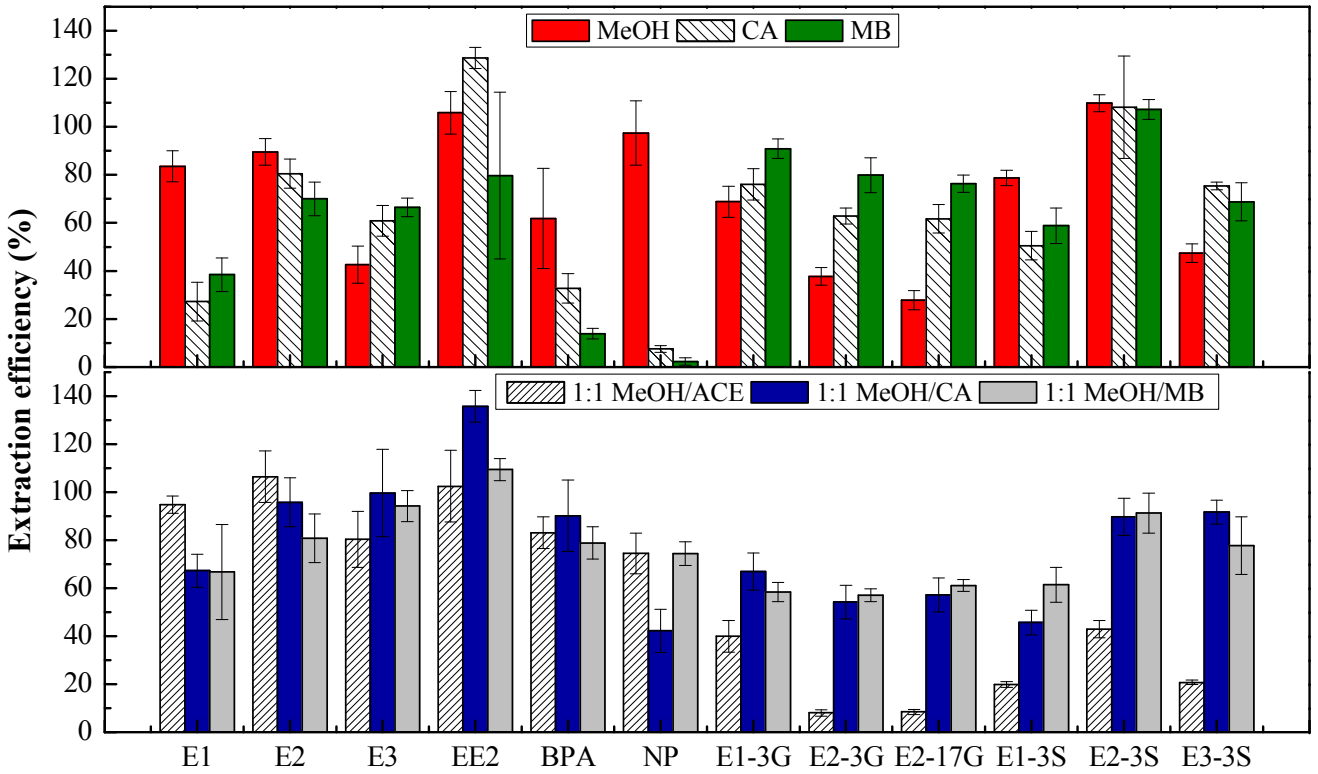


Fig. 3. Extraction efficiencies of different solvents for 12 target EDCs in the ultrasonic solvent extraction process (ACE: acetone; CA: citric acid; MB: McIlvaine buffer; MeOH: methanol).

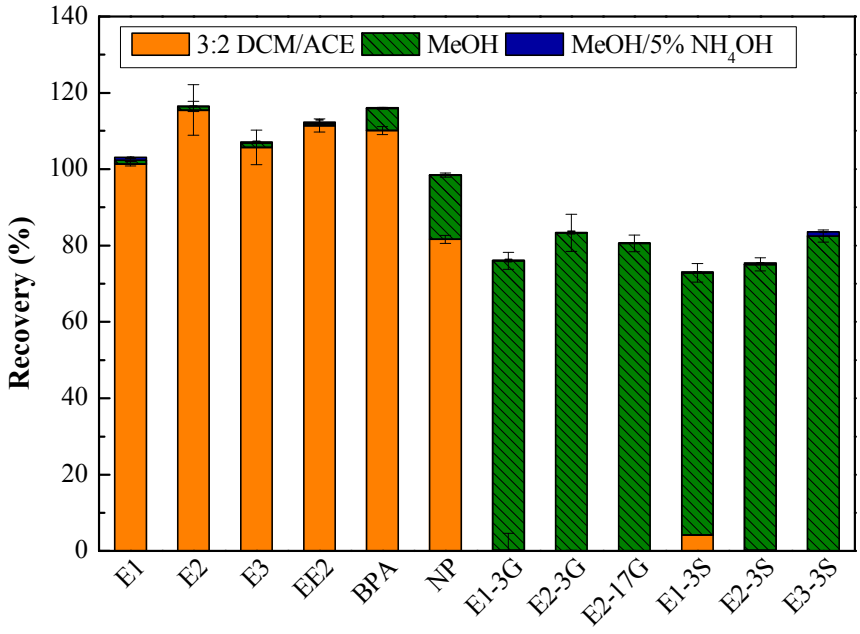
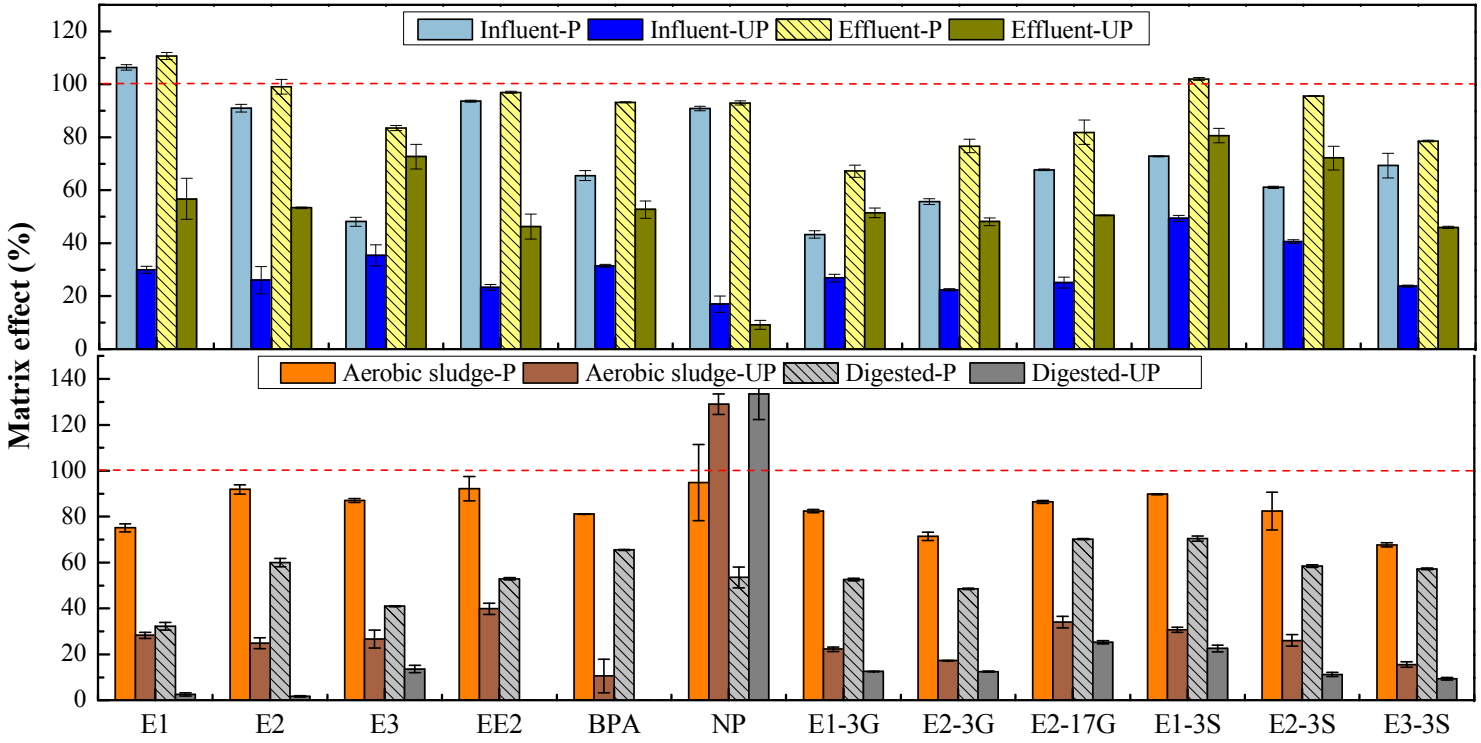


Fig. 4. Cumulative recoveries of sequential elutions with different solvents for 12 target EDCs in the solid-phase extraction process (ACE: acetone; DCM: dichloromethane; MeOH: methanol).



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