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Extraction of natural estrogens in environmental waters by dispersive multiwalled carbon nanotubes-based agitation-assisted adsorption and ultrasound-assisted desorption

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Abstract

A dispersive multiwalled carbon nanotubes (MWCNTs) based agitation-assisted adsorption and ultrasound-assisted desorption method has been developed for extracting natural estrogenic steroids in environmental waters prior to high performance liquid chromatography-diode array detection (HPLC-DAD) analysis. With the aid of agitation, the trace estrogens in bulk aqueous solution were adsorbed onto the milligrams of dispersive MWCNTs. After filtration, the estrogens enriched on MWCNTs nanoparticles were quickly desorbed into milliliters of organic solvent in ultrasonic bath. Finally, the analytes in organic solvent were directly determined by HPLC-DAD. The stability examination of estrogens under the tested ultrasonic irradiation was performed and the results indicated that the estrogens were stable during the extraction process. Several parameters that could influence the extraction efficiency, such as type and volume of desorption solvent, consumed amount of MWCNTs, pH and ionic strength of sample solution, agitation-assisted adsorption and ultrasound-assisted desorption time, were examined. Under the optimized conditions, the recoveries for three analytes in spiked samples were over 82%. The detection limits were 0.076 ng mL^{-1} for estrone, 0.049 ng mL^{-1} for 17β-estradiol, and 0.057 ng mL^{-1} for estriol, respectively, while the relative standard deviations were less than 9% (at 20 ng mL^{-1} estrogens level for 10 runs).

Keywords: Multiwalled carbon nanotubes; Dispersive solid-phase microextraction; Estrogens; Agitation-assisted adsorption; Ultrasound-assisted desorption

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1 Introduction

Estrogenic steroids, one of the most important groups of endocrine-disrupting compounds (EDCs), are considered as priority pollutants in environmental waters due to their potential adverse effects on aquatic wildlife and human health by disrupting growth, development, and reproduction even at ultra-trace levels [1-3]. The analytical techniques to efficiently enrich and analyze estrogens at low concentrations in aqueous matrices are essential for extensive surveys on their occurrence and fate in the environment. Conventional sample pretreatment techniques such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE) prior to chromatographic determination have been widely used for extraction and preconcentration of the estrogens in environmental samples [4-7]. While reliable, these methods have several shortcomings such as lengthy assay time and limited enrichment factors. Moreover, the use of large volumes of organic solvents gives rise to large amounts of organic wastes, resulting in environmental and safety concerns. In the past decades, a large number of microextraction methods such as liquid-phase microextraction (LPME) [8-9], solid-phase microextraction (SPME) [10-11], single drop microextraction (SDME) [12], which are more sensitive, cost-effective and environment friendly comparing to conventional extraction methods, have been successfully developed for the extraction of trace pollutants from a variety of environmental samples. Since these techniques are surface dependent processes, dispersive microextraction technique was recently proposed by means of dispersion to improve the contact area between sample solution and extractants, and further shorten the extraction time and decrease the extractant consumption [13-14]. For example, the ionic liquids-based dispersive liquid-liquid microextraction (ILs-DLLME) method was reported for preconcentrating the mercury-dithizonate chelates in water samples onto micrograms of 1-octyl-3-methylimidazolium hexafluorophosphate ($[C_8mim][PF_6]$) ILs and the

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detection limits of <0.1 μg L^{-1} were achieved for four mercury species [15]. Tsai et al. proposed the functionalized silica-based sorbents for the extraction of tetracyclines in water and milk samples by dispersive solid-phase microextraction (DSPME) method [16]. The key of those techniques is the use of highly efficient extractant media in order to maintain or even improve the preconcentration of the analytes using only a few milligrams or microliters of extractant. Therefore, updated developments in this field are mainly related to the use of new sorbent materials as extractant.

Carbon nanotubes (CNTs), both single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs), have received much attention from environmental and analytical scientists ever since their discovery due to their excellent adsorption capability and unique electrochemical properties [17-18]. A number of papers published in past decades reported that CNTs were employed as the adsorbents for removal of various pollutants from the aqueous solution [19-22]. Also, the CNTs had been successfully applied in SPE as sorbents to extract organic pollutants or heavy metals in surface water prior to instrumental analysis [23-24]. Such approaches usually led to the good sensitivity and reproducibility, as reported by Cai et al., who extract three endocrine disruptors in water samples at ng L^{-1} levels using this approach [25]. However, most of those studies were focused on the availability and extraction efficiency of CNTs or modified-CNTs as the alternative of the stationary absorbents in SPE-cartridge. A little information on the application of CNTs for SPME method is available. Wang et al. reported a novel SPME method using MWCNTs as the SPME fiber coating for gas chromatography with electron-capture detection of PBDEs in environmental samples, and high enrichment factors and low detection limits were achieved [26].

In the present study, our aims were: (i) to investigate the procedures of multiwalled carbon

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nanotubes-based dispersive solid-phase microextraction (MWCNTs-DSPME) method for precentration of the natural estrogenic steroids in aqueous solutions; (ii) to optimize the variables involved in MWCNTs-DSPME process such as type and volume of desorption solvent, amount of consumed MWCNTs, pH and ionic strength of sample solution, agitation-assisted adsorption and ultrasound-assisted desorption time; (iii) to apply the optimized MWCNTs-DSPME-HPLC-DAD method to extract and determine the ultra-trace estrogens in natural water samples.

2 Experimental

2.1 Chemicals and materials

Standards of estrone and 17β-estradiol were obtained from TCI Chemicals (Tokyo, Japan) and estriol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate, sodium hypochlorite, sodium hydroxide and hydrochloric acid were obtained from Jiani Chemicals (Wuxi, China). HPLC grade of acetonitrile, ethyl acetate, acetone and methanol were supplied by Tjshield Chemicals (Tianjin, China). Multiwall carbon nanotubes (diameter: 20-40 nm; length: 2-15 μm) were purchase from TCI chemicals (Tokyo, Japan). An ultrasound bath (AS20500BDT model, Automatic Science Instrument, Tianjin, China) was employed in all experiments. Prior to experiment, the MWCNTs were pretreated to remove amorphous carbon and metal catalysts for minimization of their effects on extraction by using a method developed by Lu and Chiu [27]. Initially, the MWCNTs were heated at 350 °C for 30 min to remove amorphous carbon. Then, approximately 2 g of MWCNTs was dispersed into a flask containing 80 ml of 70% sodium hypochlorite solution and shaken in ultrasonic bath for 20 min, and then heated at 85 °C in a water bath for 3 h to remove metal catalysts. After cooling, the MWCNTs were washed with deionized water until the solution reached neutral. Finally, the MWCNTs-

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solution suspension was filtered through a 0.45 μm nylon fiber filter (Fisher Scientific brand), and the filtrated MWCNTs were dried at 105 °C for 48 h. The pretreated MWCNTs were stored in sealed glass containers until used.

2.2 Stability test

Prior to optimizing the extraction procedures, the degradation test for interested estrogens in organic desorption solvents at concentration of 2.50 μg mL^{-1} was carried out under the ultrasonic irradiation to evaluate the stability of those analytes during the ultrasound-assisted desorption process.

2.3 Optimization of MWCNTs-DSPME procedure

The effects of type of desorption solvent on MWCNTs-DSPME procedures were firstly investigated by using the mixture of three estrogen standards. In details, 50 mg of pretreated MWCNTs was added in a glass beaker containing 250 mL of 20 ng mL^{-1} estrogens aqueous solution, and the MWCNTs in solution were dispersed in ultrasonic bath until no MWCNTs aggregates observed. After agitation at 20 rpm for 1 h using a motor agitator, the MWCNTs-estrogens suspension was filtered by 0.22 μm membrane. The filtrated MWCNTs-estrogens particles were transferred into a 5 mL centrifugation tube and 2.5 mL of organic desorption solvents was added. Then, the tube containing MWCNTs-estrogens-desorption solvent was sealed and votexed for 1 min, and placed into ultrasonic bath for 15 min. After centrifugation at 15000 rpm for 10 min, the supernatant solutions were pipetted into another tube and repeat desorption process once. Finally, the mixture of supernatants was filtrated by 0.22 μm membrane and injected into HPLC for analysis. Four organic desorption solvents including of methanol, acetonitrile, acetone and ethyl acetate were studied in this work. After selection of desorption solvent, several other key parameters, such as the volume of desorption solvent, consumed

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amount of MWCNTs, pH and ionic strength of sample solution, agitation-assisted adsorption and ultrasound-assisted desorption time were investigated and optimized to obtain the best extraction efficiency for MWCNTs-DSPME procedures.

2.4 HPLC analysis

Our previous developed HPLC-DAD method for determination of three estrogens was applied in this study [7]. In brief, an Agilent 1200 high-performance liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, a well plate autosampler, a column oven, a diode array detector, and ChemStation software was used for all experiments. The interested estrogens were separated on a Waters Symmetry C_{18} column (4.6 x 250 mm i.d., 5 μm particle size) at column temperature 30 ^{o}C by using a gradient elution program of mobile phase consisted of 3.0 mM ammonium acetate (NH_4Ac) buffer and acetonitrile (ACN) organic modifier as following: the initial elution was composed of 60% NH_4Ac buffer and 40% ACN, then the buffer decreased to 25% from 5 to 15 min and kept it for 2 min. Finally, the buffer increased back to 60% from 17 to 20 min for next run. The flow rate was 1.0 mL min^{-1}. Twenty microliters of samples or standard solutions was injected into the HPLC system. Detection of estrogenic steroids was carried out by direct UV absorbance at 205 nm. The three naturally occurring estrogens in real water samples were identified by matching retention times against those of standards and standard addition. All quantification was performed by the external calibration method based on peak areas. Calibration curves were constructed by linear regression of the peak area individual standard versus the concentration.

2.5 Sample collection and analysis

Rain samples were collected with a Teflon container or film on the grass of the Wenzhou University campus. River water samples were collected from the surface water of Wen-Rui-Tang

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River. Lake samples were obtained from the Swan Lake in Wenzhou University campus. All sites of sample collection are located in city of Wenzhou, Zhejiang province, China. After sampling, all samples were stored at 4 ^{o}C in the dark until used. Prior to the extraction, the water samples were centrifuged and filtered through 0.45 μm membrane filters to remove the impurities. The three naturally occurring estrogens in real water samples were extracted by following the optimized MWCNTs-DSPME method and determined by HPLC-DAD. All sample containers, glassware and filtration devices were thoroughly cleaned with 0.1 M HCl solution and then finally rinsed with doubly distilled-deionized water.

3 Results and discussion

3.1 Stability of estrogens under the ultrasonic irradiation

The stability of interested estrogens under the tested ultrasonic conditions was investigated by their concentrations time-course study in organic desorption solvents, which were used in this study. As presented in Table 1, all three estrogens were stable up to 3 hours in the presence of ultrasound at the used frequency and power in all solvents. Therefore, the ultrasound can be employed as enhancement approach for the estrogens desorption from MWCNTs without any negative influences on the target analytes.

3.2 Optimization of MWCNTs-DSPME procedure

The interactions including hydrophobic effect, π-π bonds, hydrogen bonds, and covalent and electrostatic interactions have been observed and are responsible for the adsorption of organic chemicals on CNTs sorbents. These interactions, their strengths, and contribution to the overall sorption process were comprehensively reviewed by Yang and Xing [41]. Due to the nonpolar bonds and high aspect ratios (length to diameter ratio), the MWCNTs are insoluble in

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water and easy to result in aggregation and bundling [28]. This fact diminishes the real surface area and affects their adsorption capability. Thus, the disaggregation of MWCNTs is essential before extraction. Ultrasonic irradiation was applied in this work for disaggregation and initial dispersion of MWCNTs in sample solution. Then, the ultrasound bath was turned off during the agitation-assisted adsorption process since the ultrasonic irradiation could inhabit the adsorption of the estrogens onto MWCNTs. The factors affecting the adsorption and the subsequent desorption from MWCNTs into organic solvent were studied.

3.2.1 Type and volume of desorption solvents

In this study, the type and volume of desorption solvents on desorption of estrogens from MWCNTs were investigated firstly. As opposed to aqueous solutions, hydrophobic estrogens absorbed on the MWCNTs are expected to be desorbed into organic solvents, especially under the ultrasonic irradiation. Four common organic solvents including of methanol, acetonitrile, acetone and ethyl acetate were evaluated for estrogen desorption. As illustrated in Fig. 1, the acetone yields greater desorption efficiency than the methanol, acetonitrile, and ethyl acetate solvents for all three estrogens. The reason for this could be that the solubilities of estrogens in acetone are higher than those in the other three solvents [29]. For saving the organic solvents used, the different volumes of acetone including of 2.50, 2.00, 1.50, 1.25, 1.00 mL were used for desorbing estrogens from MWCNTs separately. As shown in Fig. 2, the compatible results were achieved when the volume of acetone decreased as less as 1.25 mL. Therefore, 1.25 mL of acetone was selected as the desorption solvent in the following experiments.

3.2.2 Amount of MWCNTs

The effects of amount of MWCNTs on adsorption of target estrogens from aqueous solution were studied. The experiments were performed by adding different amounts of MWCNTs

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adsorbents (5, 10, 25, 50, 75 and 100 mg) in 250 mL of 20 ng mL^{-1} estrogens aqueous solution and following the procedures as described in section 2.3. As presented in Fig. 3, the HPLC peak areas of analytes slightly increased when the amount of the MWCNTs increased from 5 to 10 mg. This is easy to be explained that more adsorbents would possess larger surface area and more active sites for adsorbing estrogens since the available active sites of the MWCNTs are mainly the cylindrical external surface [30, 31]. However, the peak areas decreased when the amount of MWCNTs increased from 10 to 100 mg. The reason might be that the more MWCNTs-estrogens aggregates produced after filtration with the increasing amount of MWCNTs used and those estrogens inside the aggregates could not be easily desorbed into small volume of organic solvents. The larger volume of desorption solvents could result in the more estrogens desorbed. However, for reducing organic solvents consumed and considering the enrichment factors of extraction, 10 mg was chosen as the optimum amount for MWCNTs absorbents used based on the experimental results.

3.2.3 pH of sample solution

For the investigation of pH effect, a series of estrogens solutions with the pH values of 2, 4, 6, 8, 10 and 12 were prepared by adding 0.01M HCl and NaOH solutions prior to extraction. As presented in Fig. 4, changing the solution pH from 2 to 10 did not affect the adsorption of three estrogens, but there was an observable negative influence on the adsorption with an increase of pH from 10 to 12. The increase of solution pH over the pKa of the estrogens, which are ranged from 10 to 11 [32], led to increased their ionization and hydrophilicity, and thus decreased adsorption due to reduced hydrophobic interaction. Also, the negatively charged MWCNTs-OH^- could be formed in strong basic solution and the electrostatic repulsive forces between MWCNTs-OH^- and estrogen anions would further inhibit the adsorption of estrogens. Moreover,

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231 the formation of water clusters and reduction of H-bonding could decrease the adsorption affinity
232 between hydrophobic compounds and MWCNTs [33, 34]. Therefore, the MWCNTs-DSPME
233 method can be directly applied for estrogens extraction from environmental water samples
234 without adjusting pH since the typical pH values of natural waters are located in the range from 5
235 to 9.

236 ***3.2.4 Ionic strength of sample solution***

237 Generally, the solubility of the hydrophobic compounds decreases with increasing ionic
238 strength in aqueous solution. This "salting-out" effect may slightly enhance their hydrophobic
239 interactions with MWCNTs [35]. On the other hand, the aggregation of CNTs could be enhanced
240 by the increase of ionic strength, namely "squeezing-out" effect, since the repulsive force
241 between the CNTs would become smaller due to the penetration of the counter-ions into the
242 diffuse double layer surrounding the CNT particles [34]. The aggregation structure of MWCNTs
243 is unfavorable for estrogens adsorption. To examine the impacts of ionic strength, experiments
244 were performed by addition of NaCl salt in water samples from 0 to 6% prior to extraction. As
245 shown in Fig. 5, increase of ionic strength had negligible effect on the adsorption of estrogens by
246 the MWCNTs, suggesting that within the ionic strength range studied, the contribution of
247 salting-out effect to estrogens was equivalent to that of the squeezing-out effect to MWCNTs or
248 both the salting-out effect and squeezing-out effect were too weak to exert any change in the
249 adsorption of estrogens on the MWCNTs. Thus, ionic strength of natural water samples is not
250 expected to exert a significant effect on the adsorption of estrogens by MWCNTs.

251 ***3.2.5 Agitation-assisted adsorption and ultrasound-assisted desorption time***

252 The agitation may accelerate the adsorption of hydrophobic compounds onto the solid
253 adsorbents in aqueous solution due to the increase of their collision probability. On the other

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hand, the desorption processes are also favored by increasing mass transfer and diffusion, which can be significantly strengthened by ultrasonic irradiation [36]. Thus, agitation and ultrasonication have been employed as adsorption and desorption enhancement tools in sample microextraction procedures, respectively. For saving extraction time, the agitation-assisted adsorption time and the ultrasound-assisted desorption time were optimized by performing the time-course study following the described MWCNTs-DSPME procedure. As illustrated in Figs. 6 and 7, 20 min of adsorption and 5 min of desorption or longer resulted in the compatible results with the aid of agitation and ultrasonication, respectively.

3.3 Method evaluation

The estrogens in extracts of standards and environmental water samples were identified by matching retention times against those of standards and standard addition. All the calibration standards and the extracts of samples were run in triplicate. The mean values of retention time for E1, E2, and E3 were 11.21, 8.77 and 3.28 min, respectively, with the relative standard deviation (RSD) value of 0.5%. All quantification was performed by the external calibration method based on peak areas. Standards mixtures of the three estrogen compounds in the concentration range of 0.00 - 15.0 μg mL^{-1} were prepared for calibration curves. Calibration curves were $y = 65.3 x – 5.18$ for E1, $y = 81.4 x – 5.44$ for E2, and $y = 106 x – 8.66$ for E3 (y is the ratio of peak area of standards; x is the concentration of standards). All of them were linear over the concentration ranges tested with correlation coefficients over 0.999. The detection limits measured as three times the background noise for the developed MWCNTs-DSPME-HPLC-DAD method were 0.076 ng mL^{-1} of E1, 0.049 ng mL^{-1} of E2 and 0.057 ng mL^{-1} of E3, respectively, while the RSDs were less than 9% (at 20 ng mL^{-1} estrogens level for 10 runs). The enrichment factor was 175, 185, and 180 for E3, E2, and E1, respectively, based on the ratio of

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277 the volume of sample over desorption solvent and the adsorption/desorption efficiencies. The
278 described method was tested in real environmental water matrices with known amounts of three
279 estrogen standards added, and these spiked samples were subjected to the entire analytical
280 procedures from the sample pre-treatment to the chromatographic analysis. The recoveries,
281 expressed as the mean percentage ratio between the amounts found and those added, were found
282 to be 82 - 95% in different water samples, and followed the order of Rain > Swan Lake > Wen-
283 Rui-Tang River. The possible reason is that the water pollution of Wen-Rui-Tang River is much
284 worse than that of Swan Lake and the Rain is the cleanest one in three natural water samples, and
285 the soluble organic compounds in samples could significantly affect the adsorption of estrogens
286 by MWCNTs due to the direct site competition and pore blockage [34]. Compared to previous
287 reported extraction method using CNTs or modified CNTs as extractants, as listed in Table 2, the
288 amount of consumed CNTs in current DSPME method is much less than those used in previous
289 methods [25, 37-40]. Moreover, the dispersive solid-phase extraction, comparing traditional C_{18}-
290 SPE methods or using CNTs as adsorbents [5-6, 25, 40], is more efficient and environmental
291 friendly.

292 **3.4 Analysis of real water samples**

293 This developed MWCNTs-DSPME-HPLC-DAD method has been successfully applied to the
294 simultaneous determination of E1, E2 and E3 in several environmental water samples, which
295 were collected in Wenzhou, Zhejiang province, China. The typical chromatogram of Lake water
296 is presented as Fig. 8. The average concentrations of interested estrogens were 0.96 μg L^{-1} of E1,
297 1.75 μg L^{-1} of E2 and 2.65 μg L^{-1} of E3 in River and 2.32 μg L^{-1} of E1, 3.18 μg L^{-1} of E2 and
298 3.26 μg L^{-1} of E3 in Lake water samples, respectively, and all three estrogens were below the
299 detection limits in rain waters. The estrogens in Wen-Rui-Tang River water could come from the

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direct discharge of domestic sewage by local residents. The relative high concentrations of three natural estrogens in Swan Lake water may attribute to the excretion of lots of swans who living on there.

4 Conclusions

The developed dispersive MWCNTs-based agitation-assisted adsorption and ultrasound-assisted desorption method in the current study has been proved an effective, economic and environmental friendly technique for the pre-concentration of ultra-trace estrogens in environmental water samples prior to HPLC-DAD determination. It is possible to use the proposed method for extraction and measurement of other trace-level analytes in aqueous samples.

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376 **Table 1.** Concentrations of studied estrogens (initial concentration: 2.50 μg mL^{-1}) in different solvents
377 under ultrasonic irradiation for 0.5, 1.0, 1.5 and 3.0 hours

Studied estrogens	Estrone (E1)				17β-estradiol (E2)				Estriol (E3)			
Time/hour / Solvents	0.5	1.0	1.5	3.0	0.5	1.0	1.5	3.0	0.5	1.0	1.5	3.0
Methanol	2.47*	2.48	2.50	2.51	2.52	2.50	2.51	2.48	2.49	2.53	2.51	2.52
Acetonitrile	2.50	2.52	2.51	2.53	2.51	2.53	2.54	2.49	2.54	2.52	2.55	2.53
Acetone	2.49	2.52	2.52	2.53	2.53	2.52	2.54	2.55	2.54	2.51	2.53	2.55
Chloroform	2.51	2.52	2.51	2.54	2.50	2.51	2.51	2.54	2.49	2.52	2.54	2.56

378 *Units: μg mL^{-1}

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Table 2. Comparison of extraction methods based on the CNTs and other adsorbents

Samples	Analytes	Extraction methods	Consumed amount of adsorbents (g)	Estimated sample handling time (hours)	Reference
Water	Estrogens	SPE	0.50 (C_{18} silica)	2.0	[5-6]
Water	Bisphenols	SPE	0.50 (CNTs)	2.0	[25]
Butter	Estrogens	MSPE	0.40 (CNTs)	1.5	[37]
Water	Atrazine	SPE	0.10 (CNTs)	1.5	[38]
Eggs	Sulfonamides	SPE	0.08 (CNTs)	1.5	[39]
Water	Fungicides	SPE	0.10 (CNTs)	2.0	[40]
Water	Estrogens	DSPME	0.01 (CNTs)	1.0	This work

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Figure captions

Figure 1. Effects of type of desorption solvents on estrogens desorption.

Figure 2. Effects of (a) volume of acetone desorption solvents; (b) amount of MWCNTs; (c) pH of sample solution; (d) ionic strength of sample solution on estrogens adsorption/desorption efficiency.

Figure 3. Effects of (a) agitation-assisted adsorption and (b) ultrasound-assisted desorption time on estrogens extraction efficiency.

Figure 4. Typical HPLC chromatograms of (a) 10 μg mL^{-1} standards and (b) lake water after the proposed MWCNTs-DSPME extraction.

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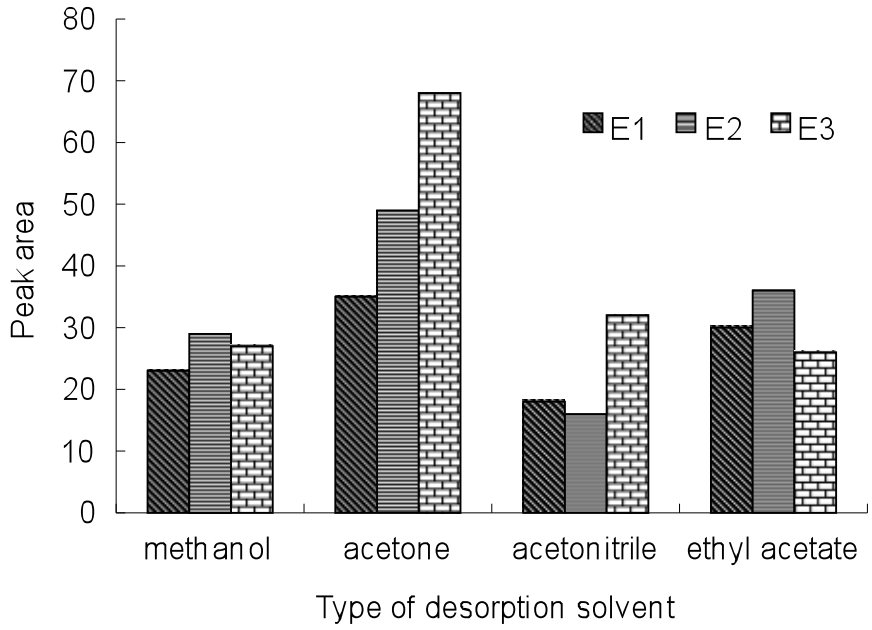

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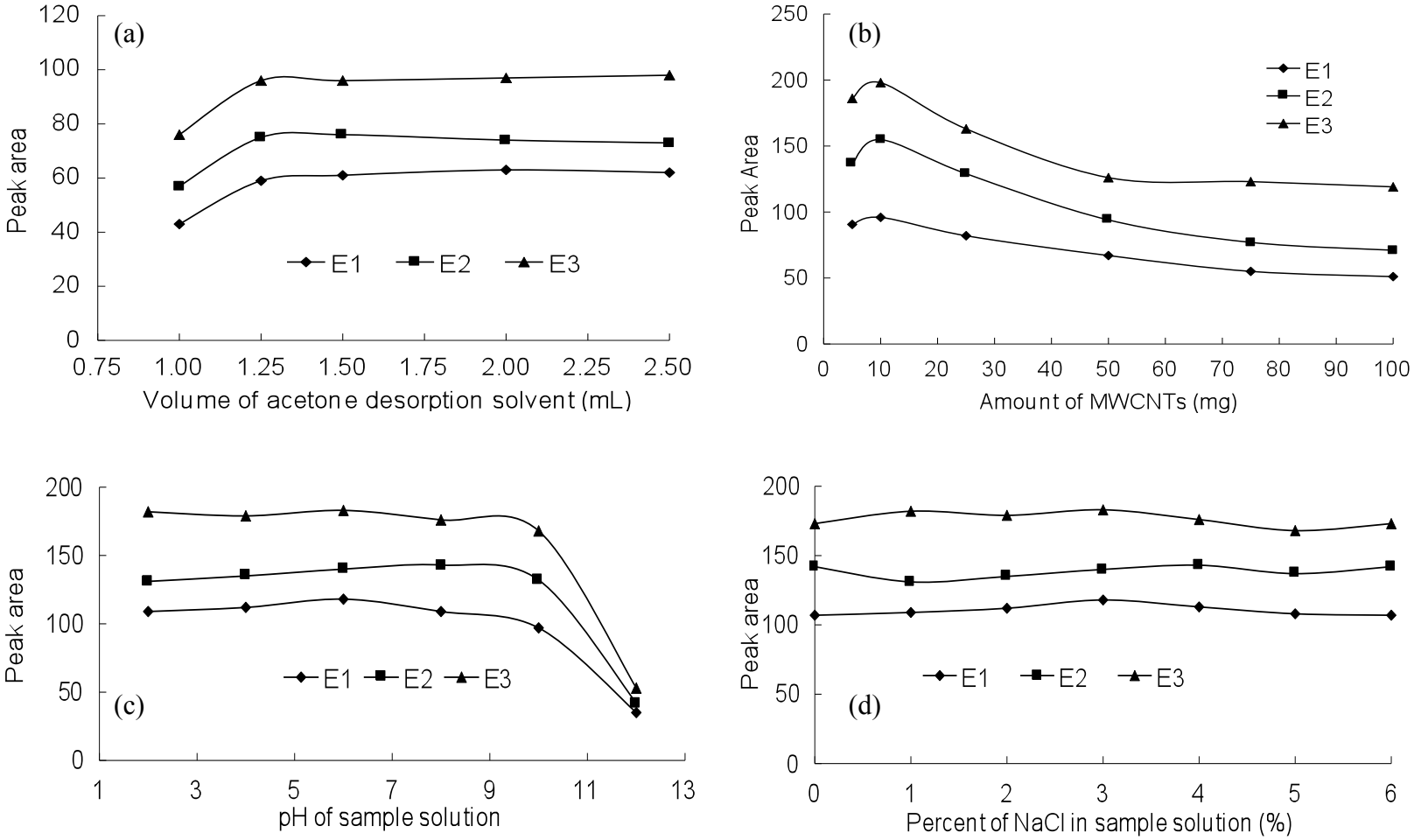

455 **Figure 2.** Effects of (a) volume of acetone desorption solvents; (b) amount of MWCNTs; (c) pH of sample solution; (d) ionic strength of sample

456 solution on estrogens adsorption/desorption efficiency.

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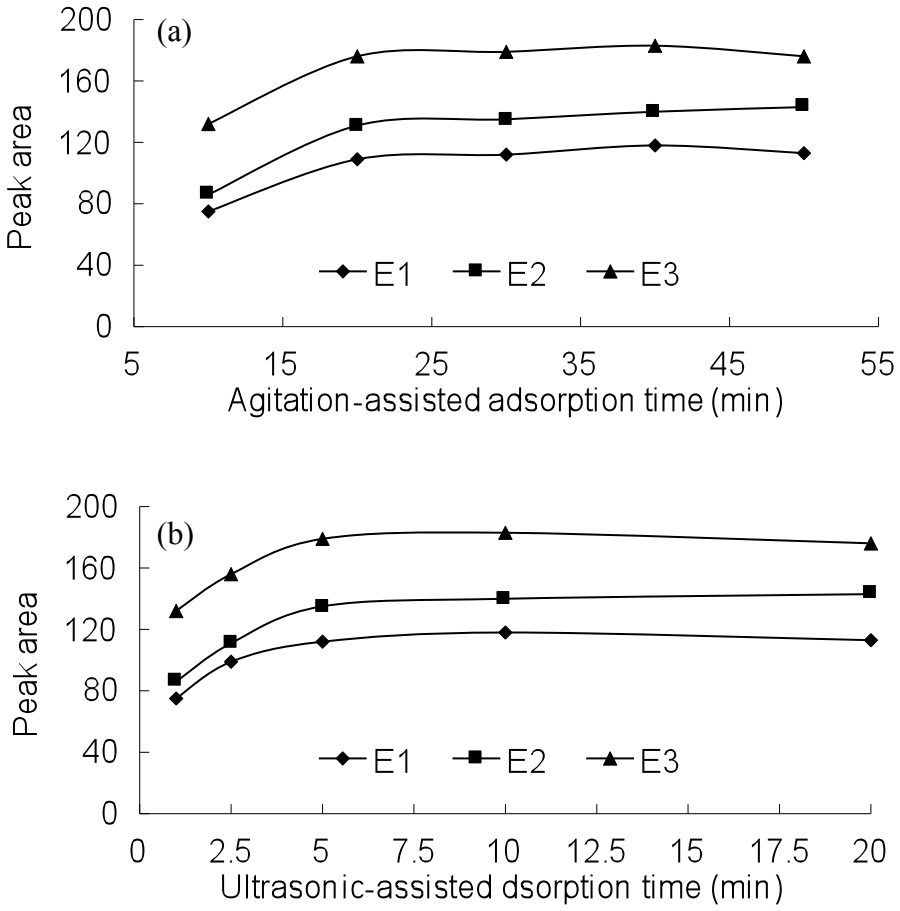

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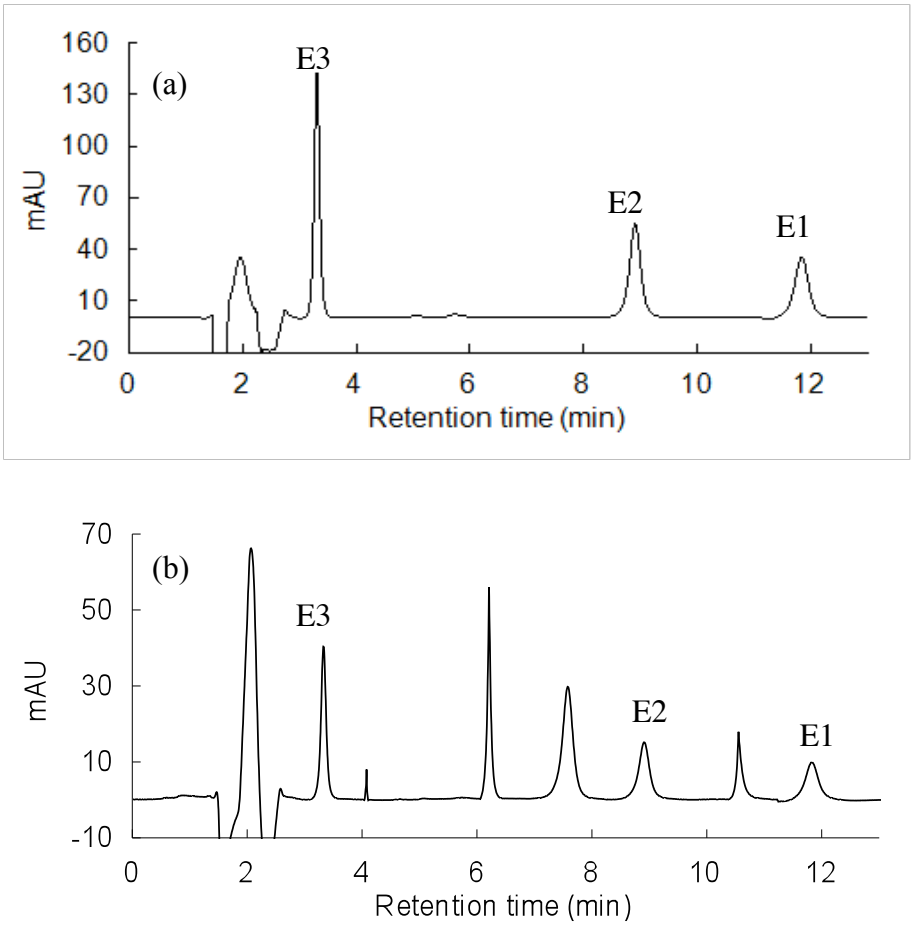

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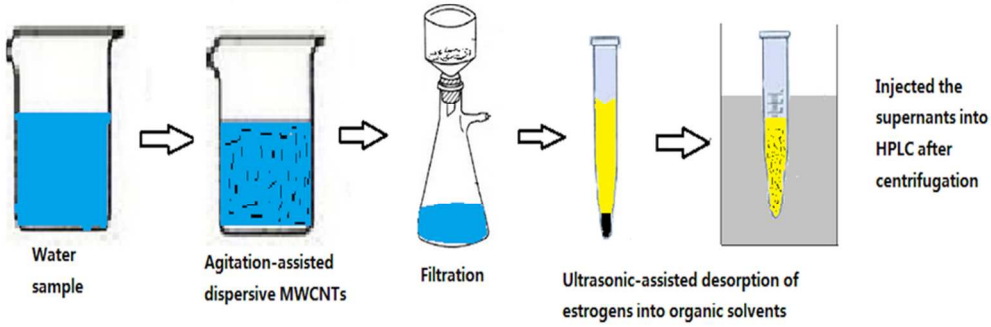

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