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Synthesis of molecularly imprinted polymer using attapulgite as matrix by ultrasonic irradiation for simultaneous on-line solid phase extraction and high performance liquid chromatography determination of four estrogens

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ABSTRACT

A molecularly imprinted polymer was synthesized by ultrasonic irradiation, with attapulgite as matrix using β-naphthol as the template molecule, acryloyl-β-cyclodextrin as the functional monomer, and N,N-methylenebiacrylamide as the cross-linking agent, respectively. The imprinted polymer was characterized by infrared spectroscopy and transmission electron microscopy. Compared to polymers prepared by traditional heat sources, the molecularly imprinted polymer synthesized by ultrasonic irradiation had better selectivity and faster adsorption kinetics to estriol, estradiol, estrone and diethylstilbestrol. Using the imprinted polymer as the packing material for on-line solid-phase extraction, the above four estrogens in milk samples were concentrated and analyzed. The limits of detection for these estrogens were in the range of 1–8 ng g^{-1} and reproducibility were less than 5.1% as RSDs ($n = 6$) with milk samples spiked at 100 and 1000 ng g^{-1} of each analyte.

1. Introduction

Chemistry deals with the interaction between energy and matter, and chemical reactions require some form of energy (e.g., heat, light, radiation, electric potential, and so on) to proceed [1,2]. Although precise control over chemical reactions is a key to the successful synthesis of nanostructured materials, such control is usually limited to the manipulation of various reaction parameters including time, energy input, and pressure, which are adjustable only within certain boundaries defined by the energy source employed. Each type of energy has its own realm of reaction conditions determined by its inherent reaction parameters [3]. Compared to traditional energy sources, ultrasonic irradiation provides unusual reaction conditions (a short duration of extremely high temperatures and pressures in liquids) that cannot be implemented by other methods. At present, ultrasonic irradiation is used to synthesize nanomaterials and polymers with different morphologies [4].

Molecularly imprinted polymers (MIP) provide an effective method for molecular recognition via the template-directed synthesis of highly crosslinked polymeric matrices [5], which has been proposed in recent years as sorbent for the extraction and/or removal of endocrine disrupting compounds [6,7]. Although MIPs prepared by conventional methods exhibit high selectivity, they still suffer some intrinsic limitations, such as the heterogeneous distribution of the binding sites, deeply embedded binding sites in bulk polymers, and poor site accessibility for the template molecule [8]. In order to overcome these limitations, MIPs based on various novel assisted-matrices have been prepared, including silica [9], multi-walled carbon nanotubes [10], chitosan [11], Fe_3O_4 magnetic nanoparticles [12], and quantum dots [13].

Estrogens have drawn extensive scientific, societal, and political attention. The prominent compounds among these substances are natural estriol (E_3), estradiol (E_2), estrone (E_1) and synthetic diethylstilbestrol (DES) (see Fig. 1). These estrogens or their metabolites play an important role in human carcinogenesis, such as breast, prostate, and ovarian cancer [14–17], and their special biological effect and potential threat to human bodies has become another hotspot. Up to now, MIPs have been widely developed for the analysis of estrogens [18–23], and most research interests focus on estrogens in environmental waters, sediment, and some aquatic product matrices, whereas studies on milk matrix have only a few reports [24–26]. For the purpose of fattening animals, large amounts of estrogens are illegally used, which can then be transported in blood, and synthesized by the mammary glands, and excreted to milk. Thus, milk is an important product

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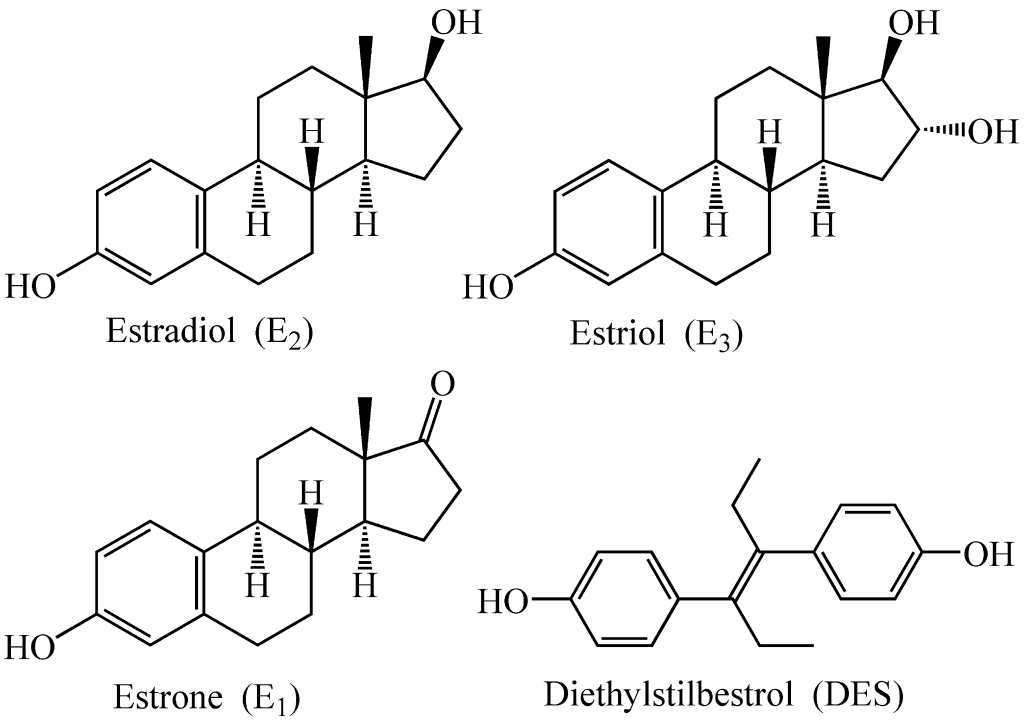

Fig. 1. Chemical structures of target estrogens.

being threatened by the use of estrogens. It has been reported that approximately 60–80% of estrogens come from milk and dairy products in Western diets [17].

Synthesizing MIPs by ultrasonic irradiation using attapulgite (ATP) as a matrix was developed in this work and is a more time-saving approach compared with the methods used in our previous studies [27,28]. The MIP synthesized by ultrasonic irradiation (SMIP) had better selectivity and faster mass transfer kinetics properties. Additionally, the SMIP–SPE column was prepared for the simultaneous on-line SPE–HPLC to determine the four estrogens in milk. The results indicated that β-naphthol SMIP exhibited the excellent recoveries for the four estrogens.

2. Experimental

2.1. Materials and instrumentation

Attapulgite (ATP) was provided by Gansu ATP (Gansu, China), which was dried in vacuum at 110 °C for 48 h before use. Methacrylic acid (MAA), ethanol, acetonitrile and acetic acid were from Tianjin Guangfu Fine Chemical Research institute (Tianjin, China). 3-Methylacryloxypropyl-trimethoxysilane (MATMS) was purchased from Alfa Aesar (Beijing, China). Estradiol (E_3), estriol (E_2), estrone (E_1) and diethylstilbestrol (DES) were from National Institute for Food and Drug Control (Beijing, China). Ammonium persulfate (APS), azo-bis-isobutyronitrile (AIBN) and 4-tertbutylphenol (TP) were obtained from Chemistry Reagent Factory of Chinese Fuchen (Tianjin, China). β-Cyclodextrin (CD) was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), recrystallized twice and dried under vacuum at 110 °C for 24 h before use. β-Naphthol was from Aladdin Chemistry Co. Ltd. (Shanghai, China). Acrylamide (AA), N,N-methylenebiacrylamide (MBAA) and N,N,N′,N′-tetra-methylethlenediamine (TEMED) were obtained from Chemistry Reagent Factory of Qian Jin (Tianjin, China). All the chemicals mentioned above were of analytical grade. The acryloyl-β-cyclodextrin (CyD) was synthesized as described by the group of Shun-ichi Nozakura and Makoto Komiyama [29,30].

Ultra pure water used throughout the experiments was obtained from the MILLI-Q (Millipore, Bedford, MA, USA) purification system. HPLC-grade acetonitrile was from Dima Technology (RichmondHill, USA).

The chromatographic analytical system consisted of a Model 210 HPLC pump and a UV detector (Varian Prostar, USA). All separations were carried out on a C_{18} column (Dikma Technologies, 5 μm, 250 × 4.6 mm). The UV–vis detector was operated at 280 nm.

The laboratory-scale sonochemical apparatus consists of a high-intensity ultrasonic titanium horn driven by a piezoelectric transducer (ultrasonic cell crusher JYD-650 from Shanghai king Industry Co., Ltd., China) which is directly introduced into a thermostated glass reactor having gas inlets and outlets.

2.2. Preparation of MIP sorbent (SMIP) by ultrasonic irradiation

Methylacryloxypropyl modified attapulgite (M-ATP) was synthesized as described in our previous work [28].

CyD (128 mg, 0.1 mmol) and β-naphthol (14 mg, 0.1 mmol) were dissolved in 25 mL ultra pure water, and ultrasound 30 min. MBAA (0.74 g, 0.5 mmol) and M-ATP (250 mg) were dispersed in the solution under stirring and then added 2 μL TEMED and 15 mg APS. Nitrogen was bubbling for 5 min and polymerization lasted for 30 min with sonochemical apparatus. After that, the polymer was collected and washed with ultra pure water and ethanol. Then template molecules were removed by Soxhlet extraction in ethanol–acetone solution (80:20, v/v) for 48 h. The process was shown in Fig. 2.

Meanwhile, the MIP synthetized by traditional sources of heat (TMIP) was prepared. Same amount of reactants as above were pooled together and then the polymerization was initiated by adding 10 μL TEMED and 15 mg APS under N_2 protection at 55 °C. The system was kept stirring for 8 h. Subsequent steps adopted the same protocol as for SMIP.

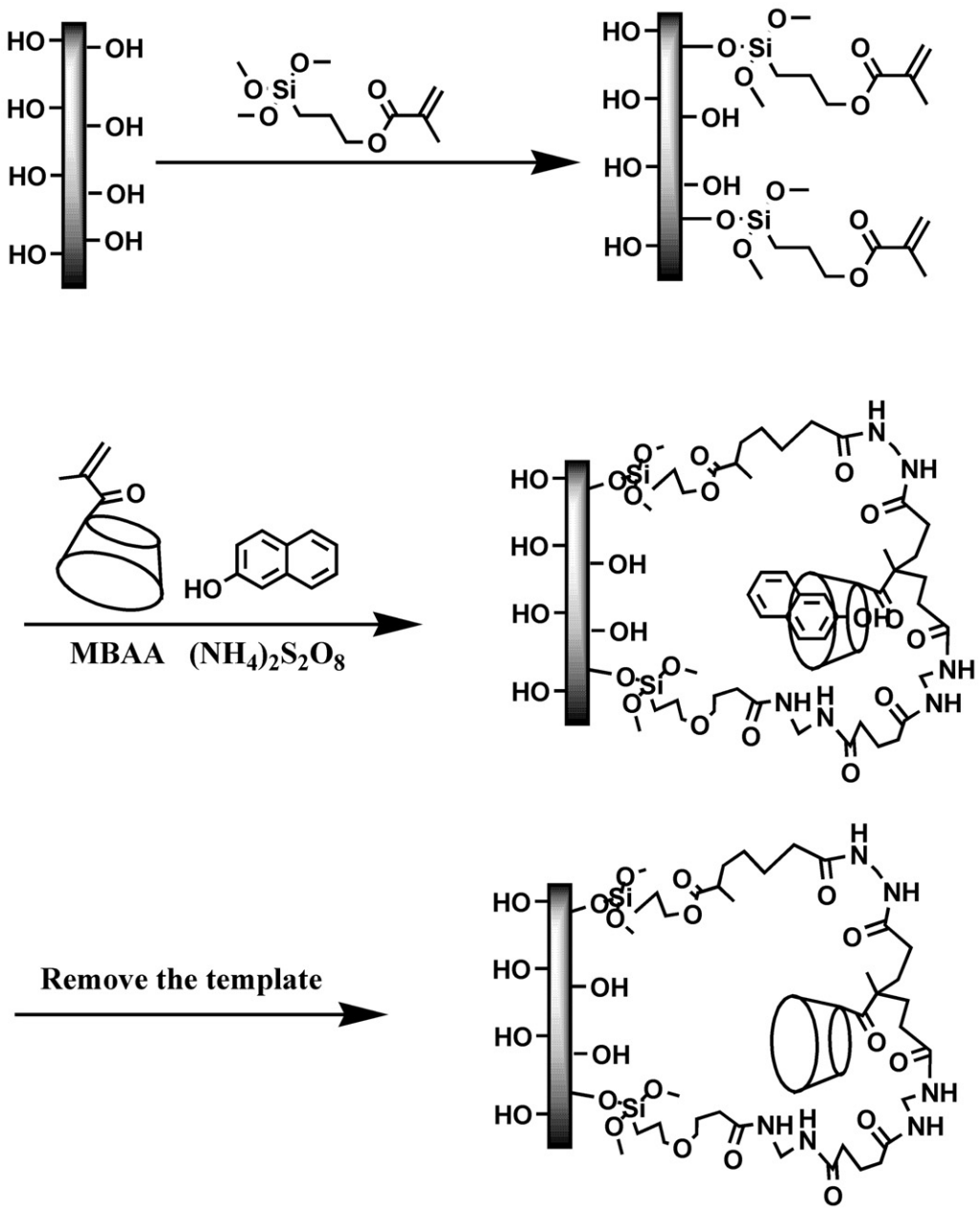

Fig. 2. Preparation procedure of molecularly imprinted polymer.

For comparison, the corresponding non-imprinted polymer SNIP and TNIP were also prepared in the similar manner except for the absence of β-naphthol.

The obtained products were characterized with Nicolet Nexus 670 Fourier transform infrared (FTIR) (MN, USA) spectrometer and JEM1200EX transmission electron microscope (TEM) (Tokyo, Japan).

2.3. Selectivity evaluation and rebinding test

Ten milligrams of the SMIP prepared by different ultrasonic frequency was equilibrated with 5 mL of E_3, E_2, E_1, DES or phenol solution (prepared with water) with concentration of 50 μg mL^{-1} to evaluate the selectivity of the imprinted sorbent. The mixture was shaken for 24 h at room temperature (about 18 °C) to facilitate the adsorption. The concentrations of free analytes were determined by HPLC. The SNIP, TMIP and TNIP were investigated in similar manner. Recognition coefficient (α) was used to evaluate the recognition ability and calculated according to the following formula (1) [31]. In theory, when α is greater than 1, it indicates that the sorbent is selective to the analyte.

$$\alpha = \frac{W_{MIP}}{W_{NIP}} \quad (1)$$

where W_{MIP} and W_{NIP} represent the amount of analyte bound to MIP and NIP sorbent, respectively.

In order to investigate the binding capacity of the SMIP, static absorption experiments were performed as followed: 10 mg SMIP materials was added into a flask and 5 mL solution of four estrogens with different concentration was added. After being shaken for 24 h in the dark at room temperature (about 18 °C), the solution were centrifuged, filtered, and then determined by HPLC. The data of the

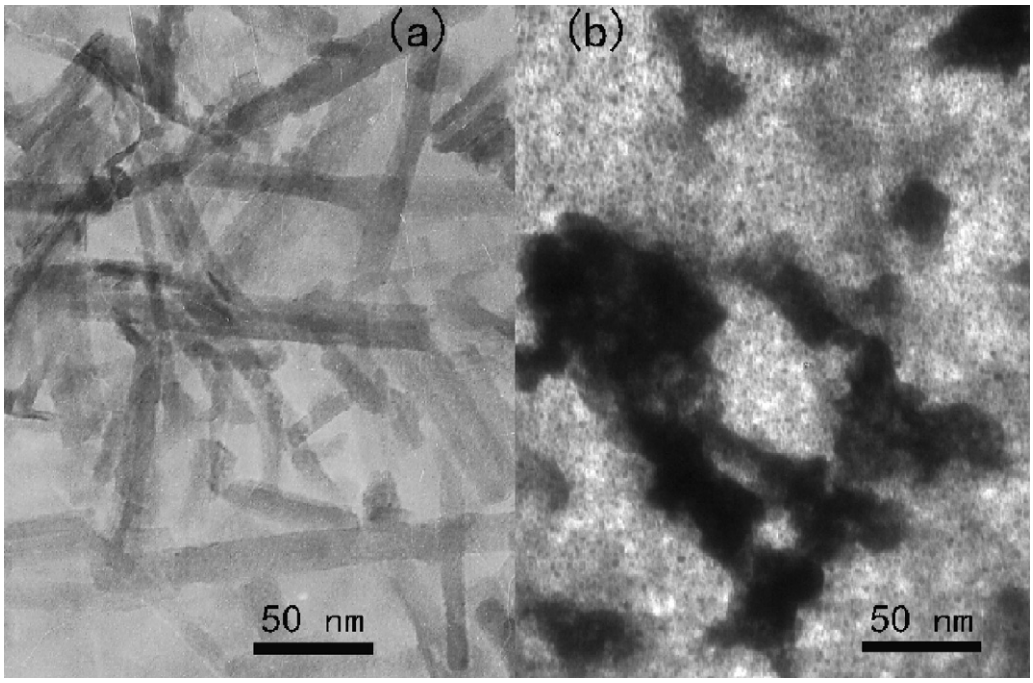

Fig. 3. TEM of ATP (a) and SMIP (b).

static absorption experiment were further processed according to the Scatchard equation (1) [32] to estimate the binding parameters of the SMIP.

$$\frac{Q}{C_{free}} = \frac{Q_{max} - Q}{K_d} \quad (2)$$

Here Q is the amount of four estrogens bound to SMIP at equilibrium, Q_{max} is the maximum binding capacity, C_{free} is the equilibrium concentration of the four estrogens and K_d is the dissociation constant, respectively.

2.4. Adsorption kinetic studies

Adsorption kinetic studies were carried out as following: 10 mg of SMIP or TMIP was suspended in 5 mL solution of E_3, E_2, E_1 and DES with an initial concentration of 50 μg mL^{-1}. The mixture was incubated at room temperature with shaking. Eight samples were taken at defined time intervals (at 5, 10, 15, 30, 60 min and 6, 12, 24 h, respectively). The residual estrogens concentrations were measured by HPLC.

2.5. On-line SPE–HPLC determination of four estrogens

In order to evaluate the applicability of the SMIP in on-line SPE determination method of four estrogens, a SPE column packed with 50 mg SMIP was prepared and placed as a six-port valve loop. The configuration of on-line SPE coupled with HPLC was same as one in our previous work [28]. Waters 510 pump was used to load samples. The SMIP–SPE column was pretreated with acetonitrile and pure water before being used.

Before the analysis of real samples, the on-line SPE conditions including sample pH, flow rate for sample loading, breakthrough volume and capacities of SMIP–SPE column were evaluated and obtained the optimal operational conditions. The extraction was performed by passing four estrogens aqueous solution through the SMIP–SPE column when the six-port injector valve set to "LOAD" position. As a result, the four estrogens were preconcentrated by the SMIP–SPE column. Simultaneously, the mobile phase was directly driven by HPLC pump through the analytical column to obtain a flat baseline. Then, the injector valve switched to "INJECT" position and the adsorbed estrogens were eluted by the HPLC mobile phase at a flow rate of 1.0 mL min^{-1} at room temperature. After 1.5 min, the six-port injector valve was again set to "LOAD" position to prepare next cycle.

2.6. Determination of four estrogens in milk samples

Different brands of pasteurized, homogenized whole milk samples were purchased from retail markets in Lanzhou, China. These samples were frozen at −20 °C until analysis.

Extraction of the four estrogens from 2.0 g spiked or original milk was carried out by first adding 2 mL acetonitrile to precipitate protein. The mixture was oscillated and centrifuged at 10,000 rpm for 15 min. The supernatant was collected and transferred to another 15 mL tube, and the residues were extracted again twice. The extract was pooled together, filtrated through 0.22 μm filter and made up to 25 mL with 15 mmol L^{-1} NH_4Ac solution. Then it was loaded on the preconditioned SMIP–SPE.

3. Result and discussion

3.1. Characteristics of molecular imprinting polymers

The TEM images of ATP and SMIP are shown in Fig. 3. There is a significant layer present on the surface of ATP, which indicates SMIP had been successfully prepared. FTIR spectra of M-ATP, CyD and SMIP are shown in Fig. 4. The characteristic peak of ATP was around 1635 cm^{-1}, corresponding to the hydrated bonds of ATP. The absorbance band of C=O at 1715 cm^{-1} in the M-ATP spectrum shows that the MATMS had been grafted onto the surfaces of the ATP. The presence of 1662 cm^{-1} and 1524 cm^{-1} in SMIP, corresponding to the hydrated bonds within CyD molecules and the C—N stretching vibration, respectively, demonstrate that the CyD had polymerized to the surface of M-ATP.

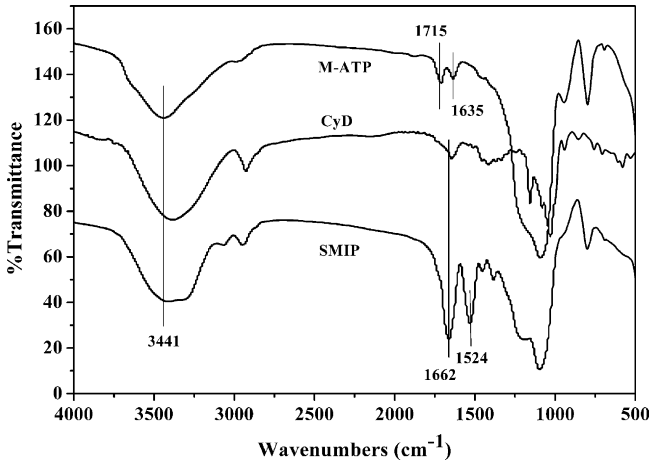

Fig. 4. FTIR spectra of CyD, M-ATP and SMIP.

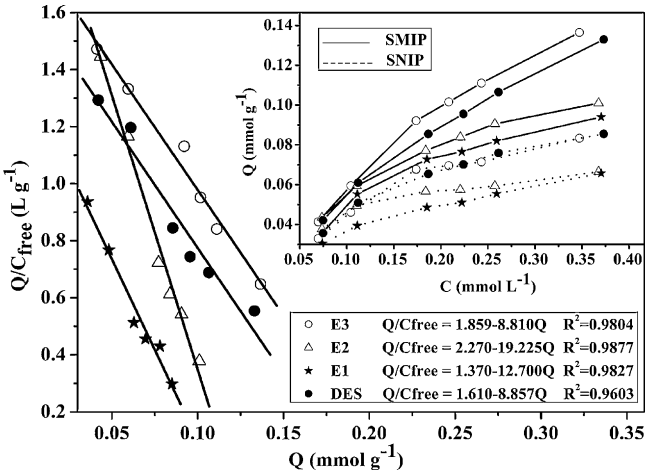

Fig. 6. Adsorption isotherm and Scatchard analysis of SMIP (each point in the isotherm was the average values of three replicates; the RSDs for all points were lower than 3.9%).

3.2. Selectivity evaluation and rebinding test of the imprinted sorbent

In order to evaluate the effect of different ultrasonic frequencies on the selectivity of SMIP, a series of SMIPs were prepared using different ultrasonic frequencies. Meanwhile the selectivity of SMIPs and TMIP to the four estrogens was compared each other. The results were shown in Fig. 5. The selectivity factors α of all SMIPs and TMIP to the four estrogens were greater than 1. Also, none of the SMIPs and TMIP could recognize the related compounds phenol, which demonstrated that the MIPs prepared using CyD as the functional monomer and β-naphthol as the template molecule had the good selectivity to the four estrogens. Meanwhile, the selectivity of the SMIPs increased with the enhancement of ultrasonic frequency. When the ultrasonic frequency reached 10–15 kHz, the obtained SMIP provided stable selectivity to four estrogens, which was slightly superior to that of the TMIP. Thus, the SMIP prepared at 15 kHz was selected for further experiments.

The SMIP material obtained at 15 kHz had higher binding capacity to the four estrogens than the corresponding SMIP (see Fig. 6). Also, only one distinct linear portion in the Scatchard analysis indicates that one type of binding site exists in the imprinted polymer, which exhibits high selectivity or affinity with high binding energy. From the slope and intercept of the straight line, K_d and Q_{max} of the higher affinity binding sites can be calculated. The K_d of E_3, E_2, E_1, and DES were 0.114, 0.052, 0.079, and 0.113 mmol L^{-1}, respectively; the Q_{max} of E_3, E_2, E_1, and DES were 0.212, 0.118, 0.108, and 0.188 mmol g^{-1}, respectively.

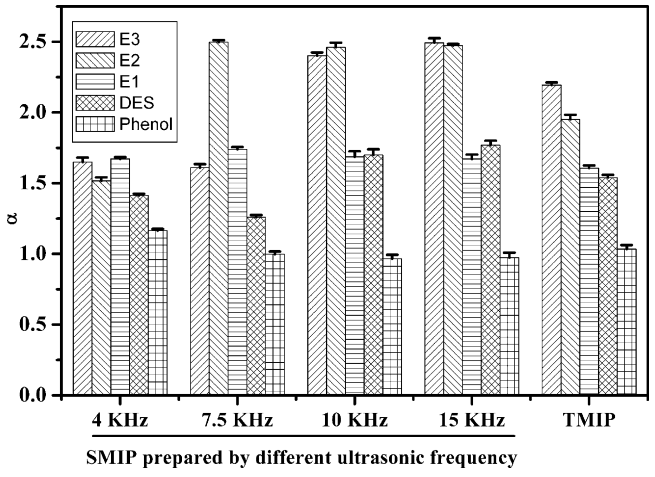

Fig. 5. Recognition coefficient of SMIP prepared with different ultrasonic frequency and TMIP to four estrogens and phenol from static adsorption test (each point in the isotherm was the average values of three replicates; the RSDs for all points were lower than 3.2%).

3.3. Adsorption kinetics

Fig. 7 shows the kinetic adsorption processes of the four estrogens at 50 μg mL^{-1} onto SMIP and TMIP. As can be seen, the adsorption equilibrium time of the estrogens on the SMIP (most at 15 min) was shorter than that on TMIP (most at 30 min). Meanwhile, the adsorption capacity of the estrogens on SMIP was higher than that on TMIP for E_3 and E_2. In order to investigate the reason that SMIP has faster adsorption kinetics and higher adsorption capacity, SMIP and TMIP were carried out with nitrogen adsorption–desorption measurements. The BET surface area, porous volume and pore size of SMIP were 212.44 m^2 g^{-1}, 0.39 cm^3 g^{-1}, and 7.69 nm, respectively. But for TMIP, the parameters were 96.97 m^2 g^{-1}, 0.11 cm^3 g^{-1}, and 5.29 nm, respectively. This may be attributed to the fact that ultrasonic irradiation could form more pores in SMIP.

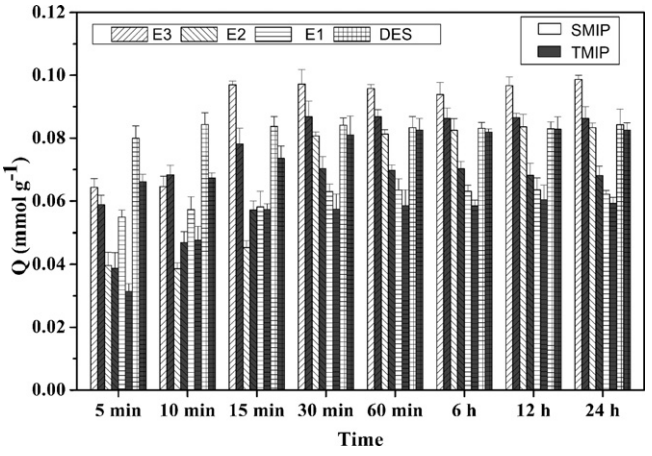

Fig. 7. Adsorption kinetic of SMIP and TMIP.

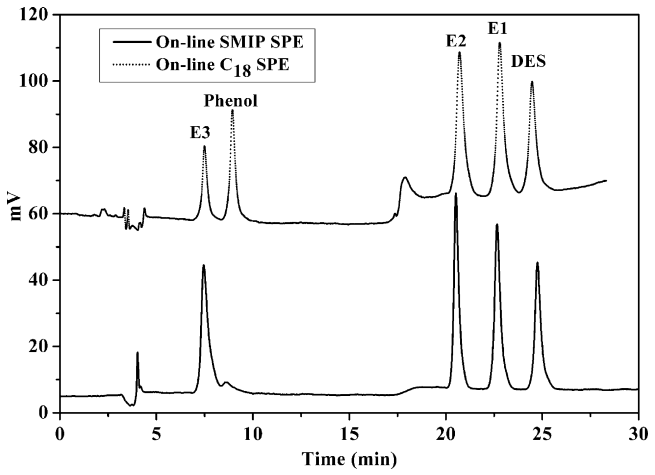

Fig. 8. Chromatograms of the estrogens after SMIP SPE and C_{18} SPE treatment. 50 mL four estrogens aqueous solution (20 ng mL^{-1}, except DES 4 ng mL^{-1}) was loaded on the SMIP SPE column at a flow rate of 3 mL min^{-1}. Chromatographic conditions: C_{18} analytical column at 30 °C (5 μm, 250 mm × 4.5 mm) and gradient elution (A: ultrapure water; B: acetonitrile; 0 min A = 60%, B = 40%; 12 min A = 60%, B = 40%; 14 min A = 45%, B = 55%; 30 min stop) with a flow rate of 1.0 mL min^{-1} and UV wavelength 280 nm.

3.4. Comparison of retention behavior of the four estrogens and phenol between C_{18}-SPE and SMIP–SPE

Chromatograms of the four estrogens and phenol through pretreated SMIP–SPE and C_{18}-SPE columns are shown in Fig. 8. It is obvious that there is no peak corresponding to phenol from the SMIP–SPE coupled HPLC system, which confirmes further that the SMIP material has good selectivity besides the similar adsorption capacity to C_{18}.

3.5. Optimization of on-line SPE process

3.5.1. Optimization of sample pH

The pH value is an important parameter influencing the capacity and selectivity of MIP in aqueous media. The effect of pH was evaluated by preparing a Britton–Robinson buffer in the range of 2–9. It was found that the four estrogens were retained well on the SMIP–SPE column in acidic and neutral solutions, which may have been due to the physical–chemical properties of the SMIP materials and the target compounds. In acidic and neutral conditions, the surface of SMIP could offer hydrogen bonding interactions with estrogenic compounds. However, at higher pH values, the four estrogens were dissociated. Therefore, the amounts adsorbed were reduced. Meanwhile, it is known that CD can easily form inclusion complexes with a guest molecule that contains phenolic hydroxyl group, and the molecule can partially or fully get into the interior of the CD cavity [33,34]. The hydrophobic cavities formed by CyDs on the surface of SMIP resulted in the hydrophobic interaction. All the interactions improved the adsorption ability of the materials.

3.5.2. Optimization of flow rate for sample loading

In order to shorten the analysis time, high flow rate was obtain by the optical sample loading flow rate, and the flow rates of 1.0–5.0 mL min^{-1} were investigated. A 50 mL sample containing the four estrogens with the concentration of 10 ng mL^{-1} (the concentration of DES was 2 ng mL^{-1}) was loaded onto the SMIP–SPE column. The recoveries were calculated by comparing the signal responses between the sample from on-line SMIP–SPE and a 20 μL standard solution with a concentration of 25 μg mL^{-1} for each compound (DES concentration was 5 μg mL^{-1}) injected directly. The results showed that flow rate had little effect on the recoveries of all analytes when the flow rate was less than 3 mL min^{-1}. Higher flow rates (≥4 mL min^{-1}) led the adsorption of E_3 and E_1 reduced, which meant that higher rates were not conducive to the full adsorption of these two analytes onto the SMIP–SPE column. In order to maximize the efficiency of the assay, the flow rate of the loading sample was chosen as 3 mL min^{-1}.

It was found that a high sample loading flow rate was feasible without the introduction of excessive column backpressure. Even when the flow rate was increased to 5 mL min^{-1}, the maximum backpressure of the SMIP SPE column was only about 500 psi. In the analysis of a real sample, the maximum backpressure increased slightly (lower than 600 psi) after several injections, which meant that the matrix impurities could be effectively flushed out of the SMIP–SPE column without clogging.

3.5.3. Breakthrough volume

Large breakthrough volume (BVI) is another advantage of the proposed method. A large BTV can improve the sensitivity and limits of detection (LOD) of the method because it provides a larger concentration enrichment factor. In this study, 30, 45, 60, 75, 90 and 105 mL of sample were investigated (the amounts of the four estrogens were kept at 1000 ng, except DES at 200 ng). The flow rate of the loading sample was 3 mL min^{-1}. The BTV of E_3, E_2, E_1, and DES were 65, 75, 90, and 90 mL, respectively. When the sample volumes applied were more than the corresponding BTV of the four estrogens, the recoveries of the estrogens declined.

Meanwhile, the capacity of the SMIP–SPE column for each analyte was investigated. In the experiment, 50 mL of 20, 40, 60, 80, 100 and 120 ng mL^{-1} estrogen solution (the DES concentrations were one-fifth that of the other estrogens) was loaded onto the SMIP–SPE column at a speed of 3 mL min^{-1}. The results showed that the recoveries of E_3, E_2, E_1 and DES dramatically declined when the concentrations of the four estrogens exceeded 80, 100, 100, and 70 (ng mL^{-1}), respectively. The corresponding maximum capacities of SMIP–SPE column was about 4.0, 5.0, 5.0, and 3.0 μg for the four estrogens, respectively.

3.5.4. Sample treatment

Because milk is complex, it is essential to remove its lipids and proteins before the SMIP–SPE procedure. In this study, acetonitrile was used for protein precipitation. The acetonitrile solution containing the analytes was further diluted to 25 mL with 15 mmol L^{-1} NH_4Ac solution to prepare the loading sample. To clean the impurities, 8 mL of a 15 mmol L^{-1} NH_4Ac water/methanol mixture (90:10, v/v) was used to wash the SMIP–SPE column at speed of 3 mL min^{-1}. The sample dilution is a way to decrease the concentration of the sample matrix and is helpful for retention of the analytes on the SMIP–SPE column. Furthermore, the NH_4Ac solution can effectively suppress the interaction of the analytes with the macromolecules in the milk sample (such as protein) and increase the solubility of the protein [35].

3.6. Method performance

Under the optimized conditions, the method performance was evaluated by the determination of the linear range, reproducibility (in terms of RSD), LODs, and limits of quantification (LOQ) of the method. The results are shown in Table 1. The LOD of each analyte was investigated from the spiked milk sample at the signal-to-noise ratio (S/N) ratio of 3:1. The LODs of all the analytes were in the low ng g^{-1} level. The accuracy and repeatability of this method were also evaluated by recoveries of spiked milk at 100 and 1000 ng g^{-1}. The recovery percentages were between 87.3 and 103.3%.

Chromatograms of the standard addition of the four estrogens in milk (100 ng g^{-1}) are shown in Fig. 9. Only DES was found in the real milk sample, and the content was 23.2 ng g^{-1}.

Table 1
Performance of on-line SPE–HPLC method for milk samples.

			E_3	E_2	E_1	DES*
Linear range ($ng\,g^{-1}$)			30–2500	25–2500	30–2500	3–500
LODs ($ng\,g^{-1}$)			8	7	8	1
LOQs ($ng\,g^{-1}$)			20	15	25	3
Intra-day	100 $ng\,g^{-1}$	Recovery	96.6	93.2	89.9	96.9
		RSD (%, $n=6$)	3.2	3.2	3.6	3.1
	1000 $ng\,g^{-1}$	Recovery	98.3	87.3	92.1	91.2
		RSD (%, $n=6$)	1.9	5.1	3.9	1.8
Inter-day	100 $ng\,g^{-1}$	Recovery	95.2	91.6	90.1	97.0
		RSD (%, $n=6$)	2.8	1.8	0.9	2.3
	500 $ng\,g^{-1}$	Recovery	90.4	98.3	93.2	97.7
		RSD (%, $n=6$)	4.8	2.3	3.2	2.6
	1000 $ng\,g^{-1}$	Recovery	101.9	93.7	91.1	103.3
		RSD (%, $n=6$)	3.2	2.8	3.6	4.8

* The spiked DES concentrations are one-fifth of the other estrogens.

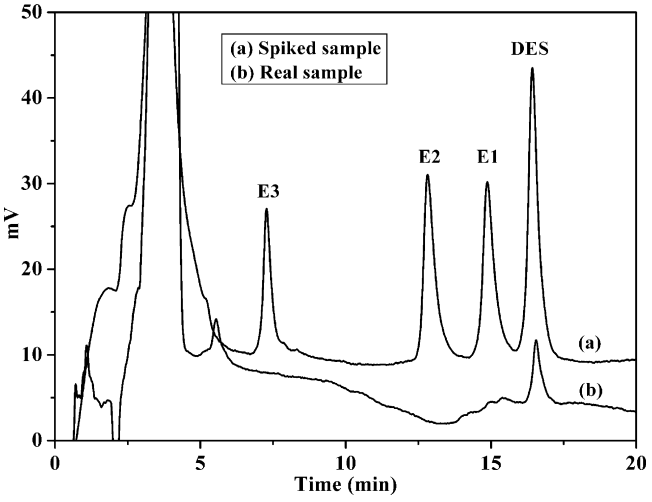

Fig. 9. Chromatograms of the four estrogens standard addition in milk (each 100 $ng\,g^{-1}$, except DES 25 $ng\,mL^{-1}$) (a), and real milk sample (b). Chromatographic conditions: C_{18} analytical column at 30 °C (5 μm, 250 mm × 4.5 mm, Dikma Technology) and gradient elution (A: ultrapure water; B: acetonitrile; 0 min A = 60%, B = 40%; 9 min A = 45%, B = 55%; 20 min stop) with a flow rate of 1.0 $mL\,min^{-1}$ and UV wavelength 280 nm.

The LODs obtained in this work are on the same order as those reported in the related literature [24,25]. Compared to the sorbent in these works, the current preparation method is more time-saving, and mimic template synthesis method can effectively avoid template bleeding. The LODs obtained in this work are higher than those of on-line C_{30} SPE coupled with HPLC–MS method [26], but with better selectivity to the four estrogens.

4. Conclusion

In this paper, β-naphthol imprinted material with ATP as the matrix has been prepared successfully by ultrasonic irradiation with a short preparation time, which was only 30 min. compared with TMIP synthesized by traditional heat method, the SMIP owned higher specific recognition selectivity and faster adsorption kinetics for the studied estrogens.

The SMIP was used successfully to on-line extract four estrogens in milk samples. With the optimized conditions, the proposed method was validated by milk sample spiked with analytes mixture. This analytical method is promising to be applied for the analysis of estrogens in complex matrix with more sensitive detectors.

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