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Construction of uniformly sized pseudo template imprinted polymers coupled with HPLC–UV for the selective extraction and determination of trace estrogens in chicken tissue samples

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ABSTRACT

To assess the potential risks associated with the environmental exposure of steroid estrogens, a novel highly efficient and selective estrogen enrichment procedure based on the use of molecularly imprinted polymer has been developed and evaluated. Herein, analogue of estrogens, namely 17-ethyl estradiol (EE_2) was used as the pseudo template, to avoid the leakage of a trace amount of the target analytes. The resulting pseudo molecularly imprinted polymers (PMIPs) showed large sorption capacity, high recognition ability and fast binding kinetics for estrogens. Moreover, using these imprinted particles as dispersive solid-phase extraction (DSPE) materials, the amounts of three estrogens (E_1, E_2 and E_3) which were detected by HPLC–UV from the chicken tissue samples were 0.28, 0.31 and 0.17 $\mu g\,g^{-1}$, and the recoveries were 72.5–78.7%, 90.3–95.2% and 80.5–83.6% in spiked chicken tissue samples with RSD <7%, respectively. All these results reveal that EE_2-PMIPs as DSPE materials coupled with HPLC–UV could be applied to the highly selective separation and sensitive determination of trace estrogens in chicken tissue samples.

1. Introduction

Steroid estrogens are a large group of lipophilic, low-molecular weight, high estrogenic active compounds, which could be roughly classified as natural and synthetic estrogens. Natural estrogens (also called endogenous estrogens) include estrone (E_1), 17β-estradiol (E_2) and estriol (E_3). These compounds have been suspected of having adverse effects on the endocrine system in wildlife [1] and humans [2]. The existence of estrogens in aquatic environments has recently been reported [3,4]. It has been shown to cause the feminization of male fish at much lower concentrations ($1\,pg\,mL^{-1}$) in aquatic environment [5]. In addition, some estrogens that are used in the animal feeds promote growth rate and enhance fat deposition of animals [6]. Due to their potential carcinogenic properties and other adverse effects in human health, considerable interest was focused on developing cost-effective analytical methods for determining these compounds in environmental samples at low concentration levels [7]. A selective, accurate and sensitive analytical method for detecting estrogens in meat samples is important for the investigation of potential use of estrogens in food-safety area.

Currently, many methods have been described for the effective detecting and monitoring endogenous estrogens in animal tissues. The low concentration of endogenous estrogens, combined with the complexity of the environmental matrix, make their detection difficult, even by the most sophisticated instrumentation. Several methods for the determination of estrogens have been reported, including immunological methods [8–10], chemiluminescence [11], HPLC [12,13], LC–MS [14], GC–MS [15], which combined with sample pretreatment methods, such as solid-phase extraction (SPE), liquid–liquid extraction (LLE), accelerated solvent extraction (ASE) and supercritical fluid extraction (SFE), are the most commonly used techniques for detecting endogenous estrogens. Each method has its advantages and limitations in terms of sensitivity, selectivity and convenience of operation. Among these, SPE as a sample pretreatment method has some imperfections, since it requires large volumes of organic solvents and time-consuming operation. Dispersive solid-phase extraction (DSPE) as a new extraction method based on the SPE methodology has became increasingly popular for sample pretreatment. The sorbent is directly added into the extracts without conditioning and the clean-up is easily carried out by just shaking and centrifugation.

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This method is quick, easy, cheap, effective, rugged and safe. However, the commonly sorbents including Florisil, alumina, bonded silica sorbents and graphitized carbon black lack molecular selectivity [16–19], and the recoveries of analytes from spiked samples are low and variable. Therefore the development of stable antibody-like materials with specific binding properties for estrogens will provide novel DSPE sorbents to effectively enrich and detect trace estrogens in complicated matrices. Molecular imprinting technique coupled with DSPE was previously used by Lu et al. for the determination of chlorpyrifos in fruit and vegetable samples [20].

Molecular imprinting is an increasingly applied technique to build selective recognition sites in a stable polymer matrix, and it is also an attractive method for the preparation of selective sorbents. Molecularly imprinted polymers (MIPs) have prearranged structure and specific molecular recognition ability [21]. In the last few years, MIPs were widely used for the selective enrichment and pretreatment of target compounds existing in complex matrix [21–24]. However, the MIPs synthesized by traditional methods exhibit poor site accessibility to the target molecules due to the functionality are totally embedded by high cross-linking density in the polymer matrices [25]. In order to overcome these drawbacks effectively, the surface molecular imprinted technique has been developed [26]. With this technique, the synthesized material has more binding sites situated at the surface or in the proximity of materials surface, and shows many advantages including more effective imprinting sites, good accessibility to the target species, fast mass transfer and binding kinetics [27].

Since MIPs are synthesized using the target molecule as the template, trace amount of the template bleeding from the resultant polymer may significantly contaminate the sample, thereby deteriorating the accuracy and precision of the analysis [28,29]. To overcome this problem, a structural analogue of the target analytes can be used during MIPs design and production [30,31], and corresponding MIPs are so-called "pseudo molecularly imprinted polymers" (PMIPs). Thus, it does not matter if template bleeding due to the interested analytes can be separated from the pseudo template in the subsequent analysis.

In this study, the PMIPs for estrogens was synthesized at the surface of ~300 nm silica nanoparticles by a conventional sol–gel process using 17-ethyl estradiol (EE_2) as a pseudo template. On the other hand, based on these improvements, we used EE_2-PMIPs as DSPE materials (EE_2-PMIPs–DSPE) coupled with HPLC–UV for the selective monitoring of trace estrogens in chicken tissue samples. Encouraging results were obtained.

2. Experimental

2.1. Chemicals and reagents

Estrone (E_1), 17β-estradiol (E_2), estriol (E_3) and ethinylestradiol (EE) were all purchased from Xianju Pharmaceutical Co., Ltd. (Zhejiang, China). 4,4′-Dihydroxybiphenyl (DDBP) and aminopropyltriethoxysilane (APTES) were all purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Bisphenol A (BPA), 2,4-dichlorophenol (2,4-DCP) and tetraethoxysilane (TEOS) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

All other chemicals were of analytical grade and obtained commercially. Ultra pure water used throughout the experiments was obtained from laboratory purification system (PALL, Germany).

2.2. Sample

Chicken tissue samples were obtained from chicken farm and refrigerated at −18 °C until use.

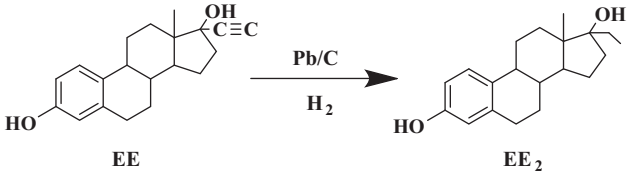
2.3. Instrumentation

HPLC was performed with a Shimadzu (Japan) system comprising LC-10ATVP pump, SPD-10AVP UV-detector, CTO-10ASVP column oven, and HW-2000 chromatographic work station. An Agilent (USA) 1200SL Series liquid chromatographic system interfaced to an Agilent 6410B Triple Quad LC–MS-MS. FT-IR spectra were recorded on a TENSOR27 infrared scanner (Bruker, Germany) with a resolution of 2 cm^{-1} and a spectral range of 4000–400 cm^{-1}. Other instruments included JEM1010 transmission electron microscope (JEOL Ltd., Japan), Thermo Scientific Heraeus Biofuge Stratos Centrifuge (Heraeus, Germany), HZ-9211KB rocking bed (Hualida Laboratory Equipment Co., Ltd.), DZG-6020 vacuum drying oven (Shanghai Samsung Laboratory Equipment Co., Ltd., China).

2.4. General procedure for the preparation of EE_2-PMIPs

2.4.1. Synthesis of pseudo template

The specific preparation method of pseudo template EE_2 was performed as follows. 2.96 g (1 mmol) EE dissolved in 15 mL ethanol was introduced into an eggplant-shaped flask containing appropriate amount of Pd/C, the reaction was carried out at room temperature under hydrogen. The product was crystallized out, filtered under reduced pressure and recrystallized three times in ethanol (95%). The final product was dried under vacuum at 60 °C over night and the following NMR and MS date were obtained: ^{1}H NMR ($CDCl_3$) δ: 0.92 (s, 3H, 18-CH_3), 1.03 (t, 3H, CH_2CH_3), 2.82 (m, 2H, 6-CH_2), 4.61 (b, 2H, OH-phenol), 6.56 (d, 1H, 4-CH), 6.63 (d, 1H, 2-CH), 7.15 (d, 1H, 1-CH). M/z: 283.2 ([M-H_2O]$^+$, 100%). The EE_2 was synthesized as the following chemical equation:



2.4.2. Preparation of silica nanoparticles

According to the report of Stöber et al. [32], silica nanoparticles were prepared by the hydrolysis and condensation of TEOS in anhydrous ethanol with ammonium hydroxide (25%) as base catalyst. Deionized water was used at every stage of the reaction. The particles were finally washed with anhydrous ethanol for three times.

2.4.3. Syntheses of EE_2-PMIPs

Uniformly sized EE_2-PMIPs were synthesized with a sol–gel process at the surface of silica nanoparticles. 50 mg of ~300 nm silica nanoparticles was dispersed in 30 mL of methanol by ultrasonic vibration. Then, 60 mg of the pseudo template EE_2 (0.2 mmol), 0.25 mL of APTES (1.0 mmol), 0.75 mL of TEOS (3.0 mmol) and 0.125 mL of HAc (1.0 mol L^{-1}) were sequentially added to the suspension under stirring. The polymerization reaction was carried out at room temperature under stirring for 20 h to obtain particles with a highly crosslinked structure. Finally, the particles were isolated by centrifugation at 10,000 rpm for 10 min and dried under vacuum at 60 °C for 12 h. As a reference, non-imprinted polymers (NIPs) for control experiment were prepared similarly as EE_2-PMIPs synthesis described above except that no pseudo template was added in the polymerization process.

To remove the template EE_2, the polymers were washed with 30 mL of mixture of methanol and 3.0 mol L^{-1} HCl (1:1, v/v) for five times, neutralized with 0.05 mol L^{-1} KOH solution and washed by deionized water until no imprinted molecule was detected in the

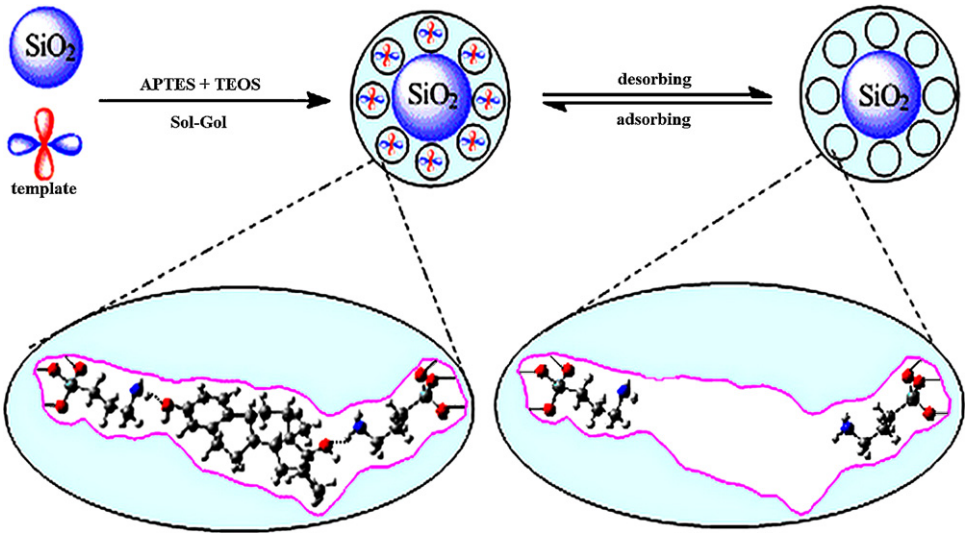


Fig. 1. Schematic representation of the possible process of EE_2-PMIPs.

rinses. Finally, the polymers were dried under vacuum at 40 °C for 12 h.

2.5. Equilibrium binding study of EE_2-PMIPs

The binding experiment was carried out by adding 20 mg EE_2-PMIPs or NIPs in a glass tube containing 5.0 mL of EE_2 standard solution which was prepared in toluene: methanol (9:1, v/v) varied in the concentration of 10–10,000 μmol L^{-1}. The solution was incubated on a rocking table for 1 h at room temperature, and then the supernatant was separated and evaluated by HPLC–UV analysis. The amount of EE_2 bound on the polymers was obtained by subtracting the free concentration from initial concentration of EE_2 added to the mixture.

The selectivity of EE_2-PMIPs was investigated using the structural analogues of EE_2 (E_1, E_2, E_3 and EE) and the reference compound (BPA, DDBP and 2,4-DCP). NIPs were used for comparison. The concentrations of each substrate solution were monitored by HPLC–UV.

2.6. Chromatographic conditions

A Varian Microsorb-MV 100-5 C18 (250 mm × 4.6 mm i.d., 5 μm particle) column was used. The mobile phase consisted of acetonitrile and water (45:55, v/v). The gradient of flow-rate was carried out starting from 0.8 to 1.0 mL min^{-1} in 4 min, held for 2 min, then to 1.2 mL min^{-1} in 2 min, held for another 2 min, and then to 0.8 mL min^{-1} in 4 min, held for 1 min. The injection volume was 20 μL. The detection wavelength was set at 230 nm.

2.7. Calibration and sample preparation

Calibration was made by the standard curve method. Stock solutions of estrogens were prepared at concentration of 5.0 μmol L^{-1} in acetonitrile. Diluting the stock solution serially with acetonitrile yielded standard solutions. All solutions were sealed and refrigerated at 4 °C until use. The calibration curve was constructed by measuring five different concentrations of estrogens ranging from 0.1 to 1.0 μmol L^{-1}. The method limits of detection (LODs) were defined as three times ratio of signal to noise.

EE_2-PMIPs–DSPE coupled with HPLC–UV was developed to determine the trace estrogens in real samples. Acetonitrile which has good ability for precipitating protein, lower solubility of lipids than other solvents and can penetrate biological tissues after homogenization was selected to extract estrogens from chicken tissue samples with ultrasonication. So 5.0 g chicken tissue samples were homogenized in 10 mL of acetonitrile, and was ultrasonicated for 30 min at room temperature. The homogenates were centrifuged at 15,000 rpm for 10 min at 4 °C. Subsequently, 1 mL of the supernatant was dried under a N_2 stream, 20 mg EE_2-PMIPs/NIPs were added to the residue, and then the complex was re-dissolved with 1 mL of toluene–methanol (9:1, v/v). After incubation for 20 min, the nanoparticles were collected, and then dispersed in 1 mL mixture solution of methanol and 3 mol L^{-1} HCl (1:1, v/v). After the nanoparticles were separated via centrifugation at 15,000 rpm for 10 min, the filtrate was dried and re-dissolved in the mobile phase (1 mL). The obtained solutions were analyzed with HPLC–UV and ascertained the unequivocal identification of target compounds in chicken tissue samples by HPLC–MS.

3. Results and discussions

3.1. Preparation of EE_2-PMIPs by sol–gel methods

In this procedure, HAc (1.0 mol L^{-1}) was used as a catalyst. TEOS was used as a cross-linking agent and designed to graft the complexes onto the surface of silica nanoparticles. The complex was formed between EE_2 and APTES, then co-hydrolyzed and co-condensed with the activated silica gel. Fig. 1 shows the possible preparation protocol of EE_2-PMIPs. The template was thought to be bound using non-covalent interaction owing to the starting functional monomers used and the template functionalities present. Fig. 1 illustrated the proposed monomer-template interaction in EE_2-PMIPs was considered as hydrogen bonding interaction. After EE_2 was removed, a large number of tailor-made cavities for EE_2 on the surface of silica nanoparticles were formed.

3.2. Characterization of TEM and FT-IR spectra

TEM images were taken for silica nanoparticles and EE_2-PMIPs. As shown in Fig. 2, the ~300 nm silica nanoparticles and EE_2-PMIPs were spherical and mono-dispersive. Fig. 2B shows EE_2-PMIPs with a size of ~320 nm. So, the imprinting layer thickness of the silica nanoparticles was estimated to be about 20 nm.

After synthesis of EE_2-imprinted silica nanoparticles, the hydrogen bonds linking the template to the matrix were cleaved

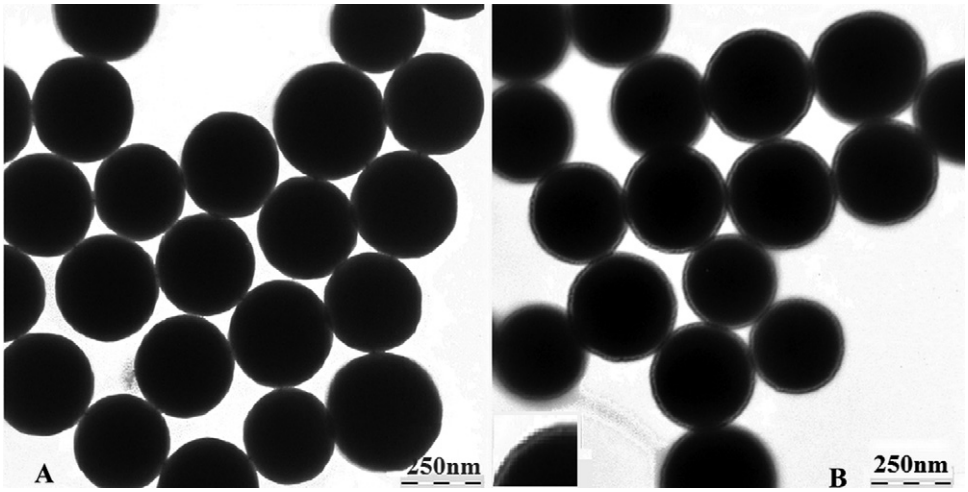


Fig. 2. TEM micrographs of: (A) silica nanoparticles and (B) EE_2-PMIPs.

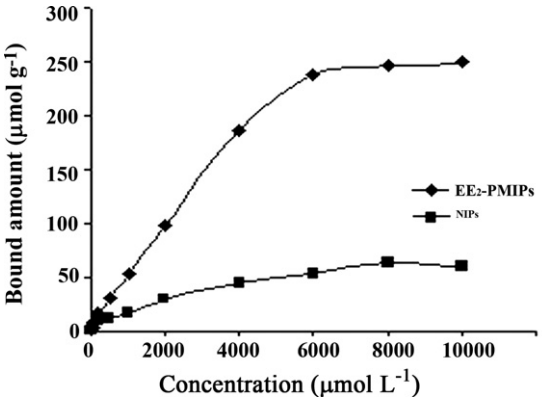


Fig. 3. The adsorption isotherms of EE_2-PMIPs (♦) and NIPs (■) to EE_2 in toluene: methanol (9:1, v/v) solutions of 10–10,000 μmol L^{-1}.

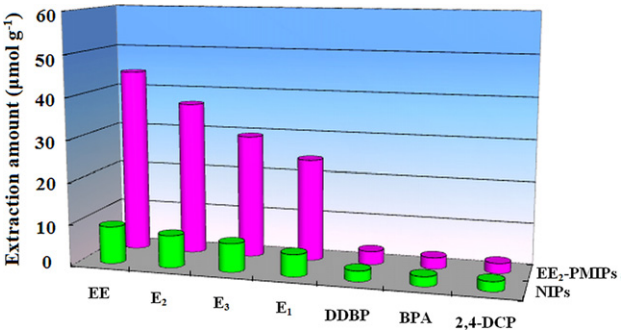


Fig. 4. The selective recognition property of each compound with EE_2-PMIPs and NIPs at the 1000 μmol L^{-1} level.

to liberate the template molecules, and consequently generated the functional amino-groups within the imprinted pocket. To ascertain the presence of amino-groups on the surface of functionalized imprinted silica nanoparticles, FT-IR spectra were obtained for silica nanoparticles, EE_2-imprinted and non-imprinted silica nanoparticles, respectively. The observed features around 1104 cm^{-1} and 980 cm^{-1} indicate Si–O–Si and Si–O–H stretching vibrations, respectively. The bands around 799 and 474 cm^{-1} resulted from Si–O vibrations. A characteristic feature of the EE_2-imprinted and non-imprinted silica nanoparticles compared with silica nanoparticles was N–H band around 1564 cm^{-1} and C–H band around 2943 cm^{-1}. This suggested that amino-groups were grafted onto the surface of silica nanoparticles after modification. Imprinted and non-imprinted polymer showed similar location and appearance of the major bands. This result is consistent with the previous report [33].

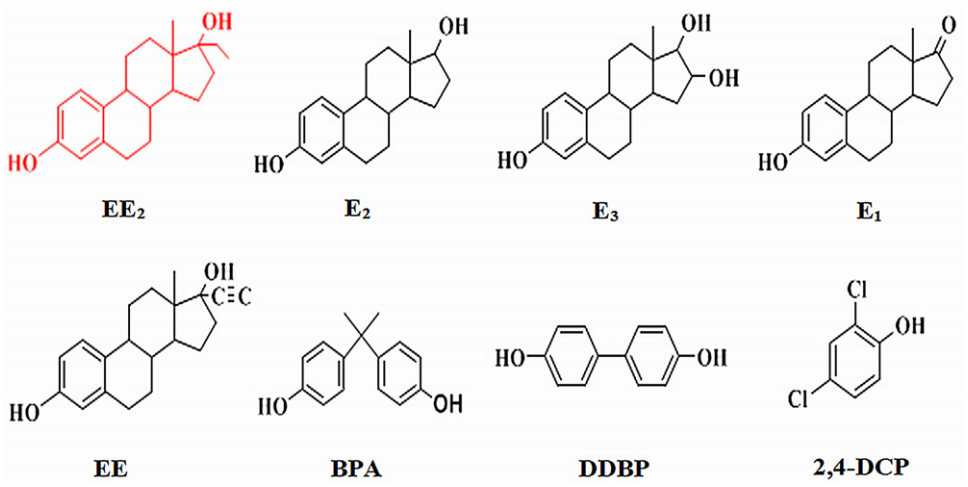


Fig. 5. The molecular structure of the compounds for selectivity investigation.

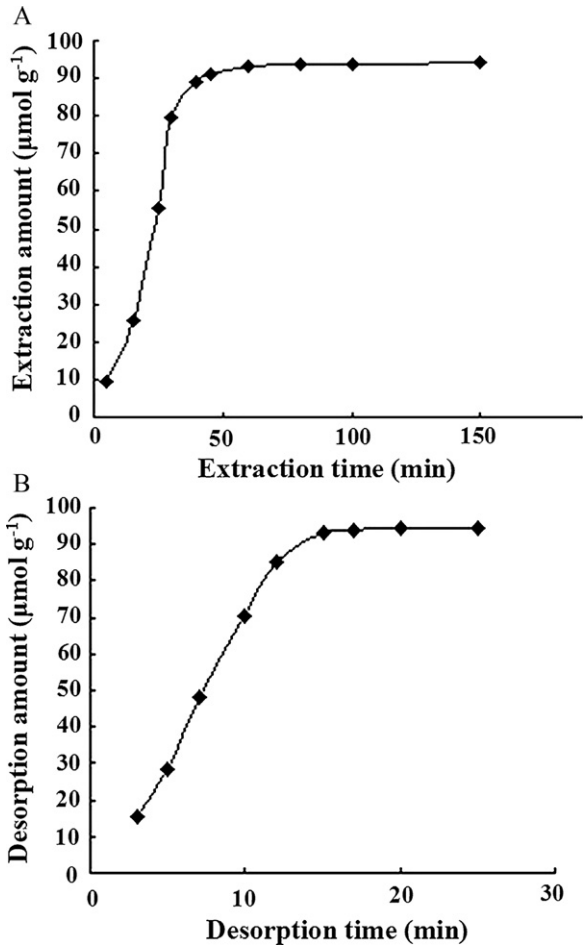


Fig. 6. Adsorption (A) and desorption (B) dynamics of EE_2-PMIPs for 2000 μmol L^{-1} EE_2.

3.3. Evaluation of the adsorption characteristic of EE_2-PMIPs

The adsorption isotherms of EE_2-PMIPs and NIPs to EE_2 are plotted in Fig. 3. It exhibited EE_2-PMIPs had a stronger memory function and a higher adsorption capacity for the template than NIPs. The adsorption capacity of EE_2-PMIPs increased with the increase of template's concentration until it reached an equilibrium state. The adsorption capacity of 20 mg EE_2-PMIPs (238.1 μmol g^{-1}) was about 4.5 times that of NIPs (53.4 μmol g^{-1}) at a 6000 μmol L^{-1} concentration of EE_2.

Successful imprinting cannot solely be evaluated on the ability of EE_2-PMIPs to rebind the template but also on its discrimination between analogue molecules. The selectivity test results among different compounds may also show some aspects of molecular recognition. Selective recognition studies were performed with each compound at a concentration of 1000 μmol L^{-1}. Fig. 4 shows the extraction amounts of some structural-related compounds and reference compounds with EE_2-PMIPs and NIPs (compounds shown in Fig. 5). The result showed that EE_2-PMIPs had high selectivity and good extraction amounts for the analogues of estrogenic compounds and poor affinity for the reference compounds such as BPA, DDBP and 2,4-DCP due to the differences in the molecular interactions and structures.

The adsorption and desorption kinetics were investigated with 2000 μmol L^{-1} EE_2. As shown in Fig. 6, the extraction and desorption reach equilibrium at about 40 min and 15 min, respectively. This short equilibrium time was mainly ascribed to the thin imprinting layer thickness of EE_2-PMIPs, and most of recognition sites of EE_2-PMIPs were produced at surface or in the proximity of the surface, so the diffusional resistance to bring the analytes into the recognition sites could be decreased.

Table 2
Determination of three estrogens (E_1, E_2, and E_3) in chicken tissue sample (n = 5).

Compound	Concentration determined in real sample (μg g^{-1})	Spiked amount (μg g^{-1})	Recovery (%)	RSD (%)
E1	0.28	0.14	75.6	5.2
		0.28	78.7	3.8
		0.42	72.5	4.8
E2	0.31	0.16	95.2	2.5
		0.32	91.0	4.0
		0.48	90.3	5.7
E3	0.17	0.08	83.2	3.7
		0.16	80.5	4.4
		0.24	83.6	6.1

3.4. Method validation and comparison with other sample preparation techniques

To validate the analytical methodology, the linearity, precision and LODs of estrogens were investigated. The results indicated that good linearity was achieved in range of 0.1–1.0 μmol L^{-1} for three estrogens (E_1, E_2 and E_3) with correlation coefficient of 0.9997, 0.9996 and 0.9997, respectively. The LODs (S/N = 3) obtained for the studied estrogenic compounds were in the range 5.4–8.1 μg L^{-1}.

The current method has been compared with other published methods concerning the determination of estrogens in Table 1. The method which was used for estrogens analysis in chicken tissue samples has good selectivity and low LOD.

3.5. Sample analysis

The established EE_2-PMIPs–DSPE coupled with HPLC–UV method was applied to analysis of trace amounts of estrogens in chicken tissue samples. The accuracy of the method was estimated by determining the sample spiked with three estrogens at three different concentration levels. As could be seen in Table 2, the amounts of E_1, E_2 and E_3 that were detected from the sample were 0.28, 0.31 and 0.17 μg g^{-1}, respectively. The average recoveries of three estrogens for the chicken tissue sample spiked with E_1, E_2

Table 1
Comparison with other published methods for the determination of estrogens by HPLC.

Extraction method	Analytical method	Sample type	LOD (μg/L)			Reference
			E_1	E_2	E_3	
Ultrasonic assisted extraction	HPLC–UV	Topic gel	10.3	19.8	NA	[34]
Liquid–liquid extraction	HPLC–UV	Estradiol HBF	1200.0	42.0	NA	[35]
C18-SPE	HPLC–UV	Water	41.0	160.0	240.0	[36]
MIPs–SPE	HPLC–fluorescence	Fish and prawn	NA	23.0	NA	[37]
EE_2-PMIPs–DSPE	HPLC–UV	Chicken tissue	8.1	5.4	7.2	This article

LOD: S/N = 3; NA: no analysis.

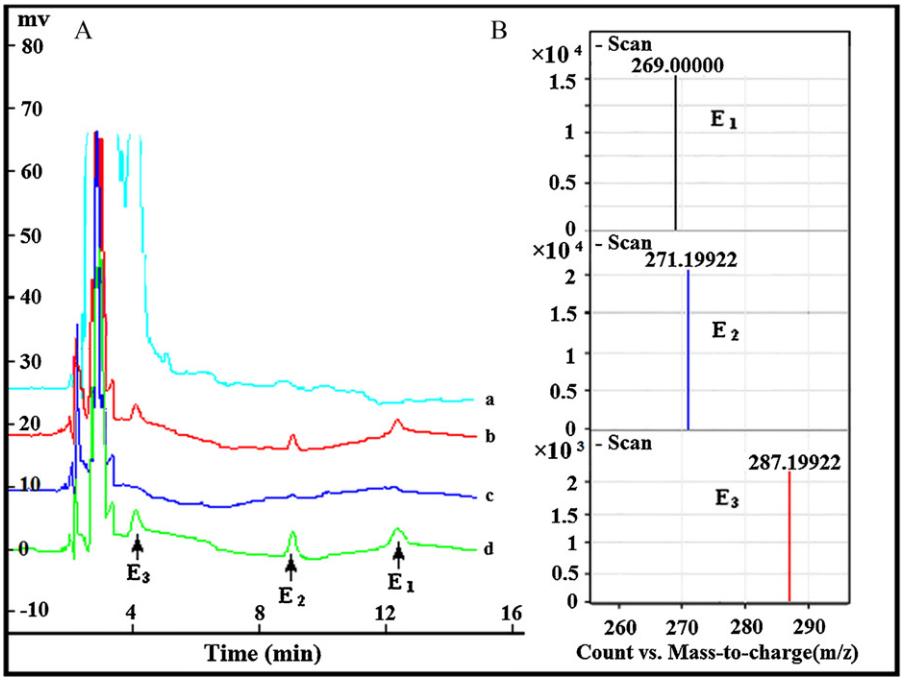


Fig. 7. (A) Chromatograms of estrogens in the chicken tissue sample (a) by direct injection, (b) with EE_2-PMIPs–DSPE, (c) with NIPs–DSPE and (d) the sample spiked with 0.28 μg g^{-1} E_1, 0.32 μg g^{-1} E_2, 0.16 μg g^{-1} E_3 with EE_2-PMIPs–DSPE. (B) Mass spectrum of E_1, E_2 and E_3 in samples by HPLC–MS.

and E_3 were 75.6%, 92.2% and 82.4% with RSD < 7%, respectively. The results demonstrated that EE_2-PMIPs had good recovery and reproducibility, and could satisfy the determination of trace estrogens in chicken tissue samples.

Fig. 7A shows that estrogens in the chicken tissue samples could not be detected by HPLC–UV without enrichment (curve a). Compared with the direct HPLC–UV analysis, estrogens are successfully accumulated and the interference is cleaned up after extracted with EE_2-PMIPs–DSPE protocol (curve b and d). The curve c shows no such selectivity with NIPs–DSPE protocol. The target compounds in the obtained solution as mentioned above have been identified by HPLC–MS (Fig. 7B).

As could be observed from the chromatograms, the sensitivities of three estrogens in the chicken tissue samples were greatly enhanced with the EE_2-PMIPs–DSPE coupled with HPLC–UV analysis. Furthermore, due to the selective binding sites at the surface of PMIPs, the PMIPs–DSPE can reach the equilibrium quickly, and this process does not require special instrumentation, consumes much less toxic organic solvent, and has a good clean-up and concentration effect for the analytes. Owing to the specific recognition of EE_2-PMIPs to the structure-related estrogens, the EE_2-PMIPs–DSPE coupled with HPLC–UV method could be applied to the highly selective separation and sensitive determination of trace estrogens in real environmental samples.

4. Conclusion

In this paper, the synthetic pseudo template EE_2 is similar with most of estrogens in shape, size and functionalities. We used EE_2 as the pseudo template to synthesize surface molecular imprinted polymers, which can accurately determine trace analytes even if some of the template was not removed in EE_2-PMIPs completely. In addition, relying on its good cross-reactivity and selectivity toward the other estrogenic compounds with the similar structure, EE_2-PMIPs were successfully used as DSPE materials to selectively enrich and determine most of estrogens in chicken samples. The results exhibited high recovery, precision and sensitivity. Therefore, those properties enabled the applications of EE_2-PMIPs–DSPE for selective extraction and sensitive screening of estrogens in real environmental samples. The proposed method also provides an effective tool for the monitoring of estrogens in food-safety area.

Acknowledgements

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