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Self-assembly molecularly imprinted polymers of 17β-estradiol on the surface of magnetic nanoparticles for selective separation and detection of estrogenic hormones in feeds

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ABSTRACT

This paper reports a surface molecular self-assembly strategy for molecular imprinting on magnetic nanoparticles for selective separation and detection of estrogens in feeds. First, γ-methacryloxypropyltrimethoxysilane (MEMO) was successfully assembled at the surface magnetic nanoparticles through simple free radical polymerization, and subsequently, the copolymerization was further assembled between methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) in the presence of templates 17β-estradiol (E2). The synthesized magnetic molecularly imprinted polymers for E2 (E2-MMIPs) showed quick separation, large adsorption capacity, high selectivity and fast binding kinetics for E2. Meanwhile, a dispersive solid-phase extraction (DSPE) based on E2-MMIPs has been established for efficient separation and fast enrichment of estrogens from the feeds. The assay exhibited a linear range of 0.1–4 μM for E2 and estriol (E3) with the correlation coefficient above 0.9996 and 0.9994, respectively. Recoveries of E2 from three kinds of feeds spiked at different concentration levels ranged from 92.7% to 97.0% with RSD < 4.7%, and recoveries of E3 ranged from 71.9% to 83.1% with RSD < 4.9%, respectively. The method is simple and sensitive, and can be used as an alternative tool to effectively separate and enrich the trace of estrogens in agricultural products by HPLC–UV.

1. Introduction

The presence of estrogenic hormones in the environment has become a concern since low concentrations (e.g., a few ng L^{-1}) of such hormones may have adverse effects on the endocrine system in wildlife [1] and humans [2]. The existence of estrogens in aquatic environments has recently been reported [3–5]. In addition, some estrogens that are used in the animal feeds promote growth rate and enhance fat deposition of animals [6]. Estrogenic hormones, such as 17β-estradiol (E2), estrone (E1) and estriol (E3), exhibit high degrees of estrogenic activity. Moreover, the synthetic estrogens, ethynylestradiol (EE) and diethylstilbestrol (DES) also have the ability to interfere with the functions of hormone systems. Due to these 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 complicated samples at low concentration levels [7]. On the other hand, because their low concentrations commonly coexisted with the complexity matrices, it is necessary to develop highly sensitive and selective methods to determine these estrogens at trace levels. The most commonly used methods include immunological methods [8], chemiluminescence [9], HPLC [10], LC–MS [11], GC–MS [12,13], which combined with sample pretreatment methods, such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), pressurised liquid extraction (PLE) and accelerated solvent extraction (ASE), etc., are the most commonly used techniques for detecting endogenous estrogens. These processes were considered complicated, time consuming, to have low selectivity, and use large amounts of organic solvents. Therefore, choosing a suitable sample pretreatment method is very important.

DSPE was recently introduced as a rapid and simple technique for clean-up crude extracts of different food and environmental samples [14–17]. It is based on the addition of the adsorbing material into the extract to remove the matrix concomitants, which is then separated from the extract bulk by centrifugation, and the technique was named as QuEChERS (quick, easy, cheap, effective, rugged and safe). However, the classical sorbents showed poor selectivity to the analytes due to the effects of complex matrices. Therefore the development of stable antibody-like materials with specific binding properties for estrogens will provide novel DSPE

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sorbents to effectively separate and enrich trace estrogens in complicated matrices. Molecularly imprinted technique coupled with DSPE was previously used for the determination of sulfonylurea herbicides in soil and crop samples [18].

Molecular imprinting is an increasingly applied technique to build selective recognition sites in stable polymers, and it is also an attractive method for the preparation of selective sorbents. Molecularly imprinted polymers (MIPs) are tailor-made polymers with a predetermined selectivity toward a given target or a group of structurally related species [19]. In addition, magnetic assisted separation is an efficient, fast and economical technology. And magnetic nanoparticles have been received considerable attention for its great potential application in magnetic bioseparation [20], targeted drug delivery [21] and enzyme immobilization [22]. Recently, there has been much research on magnetic molecularly imprinted polymers (MMIPs) for sample pretreatment. [23–27]. When magnetic nanoparticles are parts of the imprinted polymers, a unique and attractive advantage is that MMIPs cannot only selectively recognize the analytes, but also can be quickly isolated from the complex matrix by the application of an external magnetic field.

In this paper, we report a surface molecular self-assembly strategy for molecular imprinting on magnetic nanoparticles. The synthesized E2-MMIPs have quick separation, high selectivity, fast binding kinetics and good adsorption properties. Subsequently, we successfully used E2-MMIPs as DSPE materials (E2-MMIPs-DSPE) coupled with HPLC–UV for the detection of trace estrogens in feeds, and encouraging results were obtained.

2. Experimental

2.1. Materials

17β-Estradiol, estriol and ethynylestradiol were obtained from Zhejiang Xianju Pharmaceutical Co., Ltd. Methacrylic acid (MAA), acrylamide (AM), methyl methacrylate (MMA), bisphenol A (BPA), phenol (Phe), acetic acid, methanol, absolute alcohol, tetraethoxysilane (TEOS) were also obtained from Sinopharm Chemical Reagent Co., Ltd. Ammonia water was obtained from Pilot Chemical Corporation Shanghai. 2,2′-azobis(2-isobutyronitrile) (AIBN) was purchased from Shanghai No.4 Reagent & H.v Chemical Co., Ltd. γ-Methacryloxypropyltrimethoxysilane (MEMO) was obtained from Diamond Advanced Material of Chemical Inc. Ethylene glycol dimethacrylate (EGDMA) was purchased from Sigma–Aldrich Inc.

2.2. Equipment

HPLC was performed with a Shimadzu (Japan) system comprising LC-10ATVP pump, SPD-10AVP UV-detector, CTO-10ASVP column oven, and HW-2000 chromatographic workstation. Target compounds (E2 and E3) were performed using an Agilent 1200 liquid chromatographic, which was coupled to an Agilent 6410B Triple Quad mass spectrometer. Other equipment used included Milli-Q® (Millipore Co. Milford, MA, USA) water purification system, JSM5900 transmission electron microscope (TEM) (JEOL Ltd., Japan), HZ-9211KB rocking bed (Hualida Laboratory Equipment Co., Ltd.), TENSOR27 infrared scanner (Bruker Corporation, Germany), GZX-9070 MBE Electrothermal constant temperature blast oven (Shanghai Boxun Industrial Co., Ltd. Medical Equipment Factory, China), KS 130 basic rocking bed (IKA® Processing Equipment, Germany), 3K30 high speed refrigerated centrifuge (Sigma–Aldrich Biotechnology LP and Sigma–Aldrich Co., USA), C-MAG HS 7 Temperature magnetic mixer (IKA® Processing Equipment, Germany).

2.3. Calculation of binding energy

Simulation of interactions between E2 and monomers was performed using Gaussian 03 software. The initial chemical structures of E2 and functional monomer molecule were drawn using the software Gaussview2.1. The three-dimensional structure of the compounds was refined by full geometry optimization at the Hartree–Fock (HF) level of theory using the ab initio 3-21G basis set (HF/3-21G). Then, the electronic energies between template (E2) and functional monomers (MAA, MMA or AM) were calculated through semi-empirical quantum chemical methods (PM3). The binding energy (ΔE) was finally computed by the following generic formula:

$$\Delta E = |E_{(\text{complex})} - E_{(\text{template})} - \sum E_{(\text{monomer})}| \quad (1)$$

2.4. Preparation of E2-MMIPs

Fig. 1 illustrates the preparation method of E2-MMIPs. The Fe_3O_4 nanoparticles were prepared by the coprecipitation method [23], and then the resulting Fe_3O_4 nanoparticles were coated with a thin SiO_2 film: 300 mg Fe_3O_4 nanaoparticles were dispersed in 50 mL of 2-propyl alcohol by ultrasonic vibration. 2 mL of ammonium hydroxide and 2 mL of deionizer water were sequentially added under stirring, and then 5 mL of TEOS was added dropwise. After 12 h, the magnetic precipitates were isolated from the solvent by a permanent magnet and washed several times with deionizer water.

Subsequently, the magnetic nanoparticles were chemically modified with MEMO. 0.5 g of magnetic nanoparticles and 10 mL of MEMO were added into anhydrous toluene to make 50 mL of mixture solution. The mixture was refluxed for 12 h under dry nitrogen at 120 °C. The modified magnetic nanoparticles were collected by an external magnetic field and then washed with ethanol, deionizer water and toluene.

Prior to polymerization, the modified magnetic nanoparticles (40 mg) and template E2 (72 mg) were dispersed in 50 mL of toluene by ultrasonic vibration and stirred for 1 h at the room temperature. Then, the functional monomer MAA (138 mg) was added to the suspension under stirring for another 1 h. Subsequently, the cross-linking agent EGDMA (498 mg) and AIBN (30 mg) were then dissolved into the above solution. This mixture was stirred under nitrogen for 10 min. The prepolymerization was first carried out at 50 °C for 6 h, and the final polymerization was completed at 60 °C for 24 h. The products were further aged at 85 °C for 6 h to obtain a high cross-linking density. Finally, the synthesized E2-MMIPs were separated, ultrasonically cleaned by the mixture solution of methanol and 1 M HCl (1:1, v/v) and washed with deionizer water until no template was detected in the rinses. As a reference, magnetic non-imprinted polymers (MNIPs) were synthesized simultaneously under the same procedure in the absence of template molecule.

2.5. Measurements of molecular recognition properties and characterizations

A Bruker Vector 27 FT-IR spectrometer with a resolution of 2 cm^{-1} and a spectral range of 4000–500 cm^{-1} was employed to examine infrared spectra of samples by a pressed tablet (sample: KBr = 1:14 in mass).

Static binding capacity was measured by suspending 20 mg E2-MMIPs or MNIPs in 10 mL of toluene/methanol (9:1, v/v) with various E2 concentrations (0.001–6.0 mM). After the samples were incubated in a shaker for 12 h at room temperature, and then the supernatant was separated. The amount of E2 bound on the polymers was obtained by subtracting the free concentration from

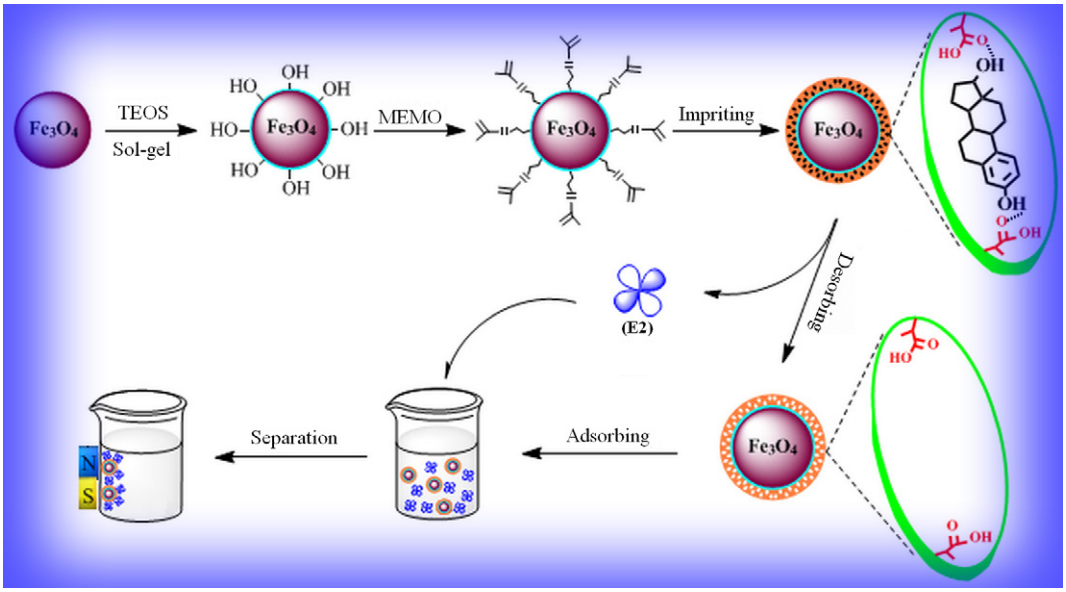

Fig. 1. Schematic representation of the possible process of E2-MMIPs.

initial concentration of E2 added to the mixture with HPLC–UV analysis. Meanwhile, the binding kinetics was tested by detecting the temporal evolution of E2 concentration in the solutions. Selective recognition studies of E2-MMIPs were performed with the structure analogous (EE and E3) and reference compounds (BPA and Phe) at a concentration of 2.0 mM.

2.6. Chromatographic conditions

The chromatographic separation was performed on an Elite Hypersil BDS C18 (150 mm × 4.6 mm i.d., 5 μm particle) column with methanol and water (75:25, v/v) mobile phase at a flow rate of 1.0 mL min^{-1}. The column temperature was 25 °C and the injection volume was 20 μL. The detection wavelength was set at 230 nm.

2.7. Method validation and application to real samples

The calibration curve was constructed by measuring E2 and E3 standard solutions of different concentrations ranging from 0.1 to 4 μM. The method limit of detection (LOD) was defined as three times ratio of signal to noise. For the assessment of the accuracy and the precision, three kinds of real samples spiked with E2 and E3 at three different levels were tested ($n = 5$).

About 1 g of pulverized feeds were first soaked with 5 mL methanol and ultrasonicated for 30 min, and the supernatant was kept. And then another 5 mL methanol was added to the remaining solids for a second extraction and the two supernatants were combined. Subsequently, 1.0 mL extract was dried under a N_2 stream and then 20 mg E2-MMIPs were added. The complex was re-dissolved with 0.5 mL of toluene–methanol (9:1, v/v) and incubated for 30 min at room temperature. The E2-MMIPs were obtained by magnetic separation, and then 0.5 mL methanol and 1 M HCl (1:1, v/v) was used as the eluted reagent to extract the analytes in E2-MMIPs. The supernatant was obtained and dried under a N_2 stream. Finally, the residues were re-dissolved in 0.5 mL of mobile phase for further HPLC–UV analysis and ascertained the unequivocal identification of target compounds in the feeds by LC–MS/MS.

3. Results and discussion

3.1. Calculation of ΔE

The selection of the suitable functional monomers is a crucial factor in the study of MIPs. In this work, three widely used functional monomers (MAA, MMA and AM) were compared theoretically. In order to evaluate the possibility of template–monomer interactions, the binding energy (ΔE) of complexes between E2 and monomers (mole ratio 1:2 for example) was calculated, as listed in Table 1. It shows that $\Delta E_{(MAA)} > \Delta E_{(AM)} > \Delta E_{(MMA)}$, indicating that the interaction of E2 and MAA is the strongest. Therefore, the E2-MMIPs synthesized with MAA are expected to give a higher selectivity to E2 than the other two functional monomers (AM and MMA).

3.2. Characterization

In order to investigate the dispersibility, stability and magnetic response, the acid resistant test was researched. In the mixture solution of methanol and 1 M HCl (1:1, v/v), Fe_3O_4 nanaoparticles and Fe_3O_4 nanoparticles with SiO_2 films were dispersed homogeneously. Fe_3O_4 nanoparticles with SiO_2 films were quickly separated by a magnet and the color of the solution did not change. Meanwhile, the solution turned yellow and this result showed that the small amounts of Fe_3O_4 nanaoparticles have been reacted with HCl. This result illustrated that Fe_3O_4 nanaoparticles coated with SiO_2 films were obtained, and stable in the acid solution.

Table 1
Binding energies ΔE of E2 with MAA, MMA and AM.

Molecules	Energy (a.u.)	ΔE (a.u.)	ΔE(kJ mol^{-1})
E2	−873.6327	–	–
MAA	−300.7518	–	–
MMA	−339.3329	–	–
AM	−242.6369	–	–
E2: 2MMA	−1552.312	0.0135	35.4
E2: 2AM	−1358.924	0.0175	45.9
E2: 2MAA	−1475.163	0.0267	70.1

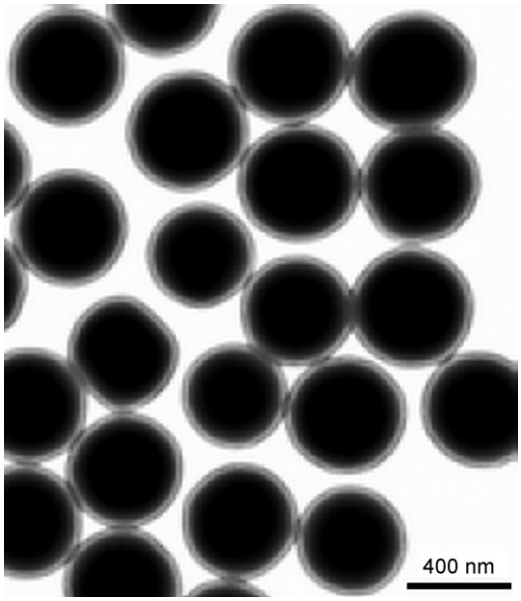

Fig. 2. The TEM image of E2-MMIPs.

The morphology of E2-MMIPs was assessed by TEM. As shown in Fig. 2, the size of E2-MMIPs is about 400 nm and the imprinting layer thickness was about 25 nm. In addition, MEMO was used as vinyl functional monomer in the experiment. To ascertain the presence of carbonylic-groups on the surface of modified magnetic nanoparticles, FT-IR spectra were obtained for modified magnetic nanoparticles and magnetic nanoparticles. The observed features around 1710 cm^{-1} indicate that C=O stretching vibration on the surface of modified magnetic nanoparticles. And the relatively strong bands at the range of 2800–3000 cm^{-1} corresponded to the stretching vibration of C–H bonds from the methyl (or methylene) groups of MEMO [28]. These data confirmed the success of chemical modification on the surface of magnetic nanoparticles.

3.3. Evaluation of the adsorption characteristic of E2-MMIPs

The molecular recognition properties of E2-MMIPs were evaluated by comparing with MNIPs. As shown in Fig. 3A, E2-MMIPs have a stronger memory function and a higher adsorption capacity for E2 than MNIPs. The adsorption capacity of E2-MMIPs increased with the increase of E2 concentration until it reached an equilibrium state. The adsorption capacity of E2-MMIPs (225.68 μmol g^{-1}) was about 4.5 times that of MNIPs (49.71 μmol g^{-1}) at a 4 mM concentration of E2. The RSD was less than 5.2% and 10.4% at each concentration of E2-MMIPs and MNIPs, respectively ($n = 5$).

To estimate the binding property of E2-MMIPs, static adsorption experiment was employed and the data were further processed

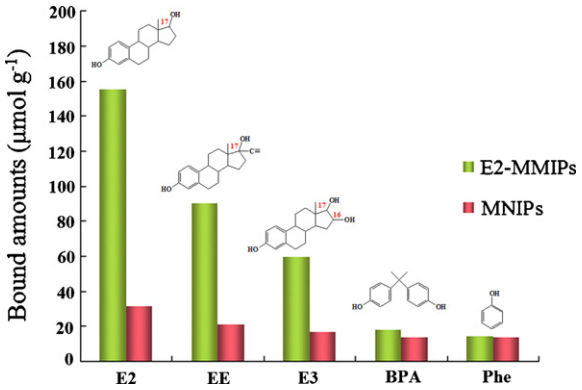

Fig. 4. The selective recognition property of each compound with E2-MMIPs and MNIPs at the 2.0 mM level.

with Scatchard analysis [29]. Binding data can be obtained by the following Scatchard equation:

$$\frac{Q}{C} = \frac{(Q_{max} - Q}{K_D} \quad (2)$$

where Q is the amount of E2 bound to E2-MMIPs at equilibrium; C is the free E2 concentration at equilibrium; K_D is the dissociation constant and Q_{max} is the apparent maximum binding amount. K_D and Q_{max} can be determined from the slope and intercept, respectively, when Q/C is plotted versus Q.

As can be seen from Fig. 3B, the Scatchard plot for E2-MMIPs was not a single linear curve, and two distinct linear portions in Scatchard analysis indicated a fact that two classes of binding sites existed in E2-MMIPs: one exhibited high selectivity or affinity with high binding energy, while another had low affinity with low binding energy. The K_D of the higher affinity binding sites was calculated to be 0.19 mM. Similarly, the K_D of the lower affinity binding sites was 6.93 mM.

The adsorption kinetics was investigated with 3.0 mM E2. As shown in Fig. 3C, the time-dependent evolution of E2 bound with E2-MMIPs and MNIPs were exhibited, respectively, and the adsorption equilibrium was reached within 30 min. The RSD of measured value with E2-MMIPs and MNIPs were less than 3.4% and 5.4% at each time point, respectively ($n = 5$).

In order to estimate the selectivity of E2-MMIPs, several estrogen compounds (EE and E3) and reference compounds (BPA and Phe) with similar structure and characteristics were selected. The results were shown in Fig. 4. The rebinding capacity of E2-MMIPs to E2 is about 1.58, 2.51, 6.00 and 7.72 times that of EE, E3, BPA and Phe at the 2.0 mM level, respectively. However, MNIPs do not exhibit the obvious difference in the rebinding capacities of E2, EE, E3, BPA and Phe. Besides this, as shown in Tables 1 and 2, the ΔE of complexes between E2 and MAA was much bigger than others. The greater ΔE shows that the interaction of compounds and MAA is better. According the experiment results, we expected that the

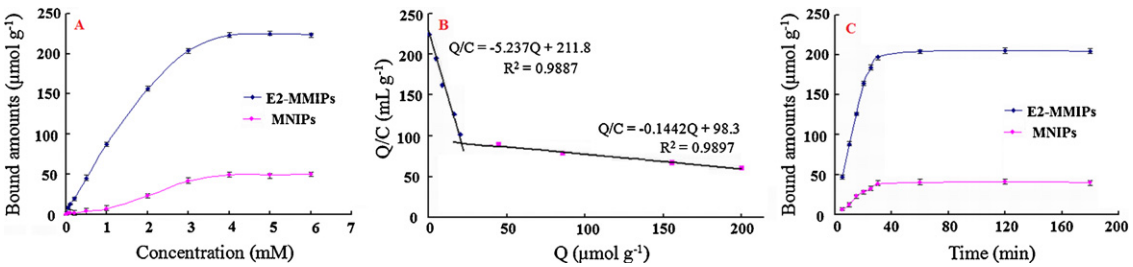

Fig. 3. (A) The static adsorption curves of E2-MMIPs and MNIPs to E2 in toluene: methanol (9:1, v/v) solutions of 0.001–6.0 mM. (B) The Scatchard plot analysis of the binding characteristics between E2-MMIPs and E2. (C) The adsorption kinetics of E2-MMIPs and MNIPs for 3.0 mM E2.

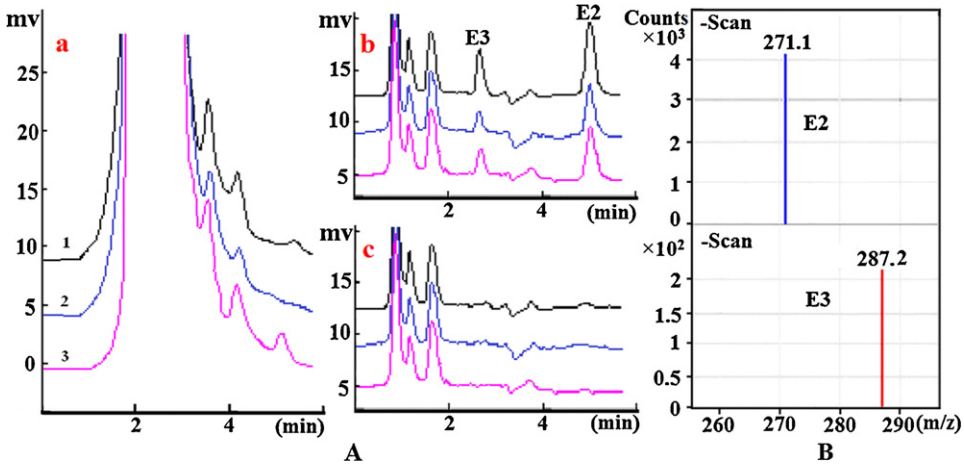

Fig. 5. (A) Chromatograms of estrogens in chicken (curve 1), goose (curve 2) or fish feeds (curve 3) with direct injection (a), with E2-MMIPs-DSPE (b), and with MNIPs-DSPE (c). (B) Mass spectrum of E2 and E3 in samples by LC–MS/MS.

Table 2
Binding energies ΔE of compounds (EE, E3, BPA and Phe) with MAA.

Molecules	Energy (a.u.)	ΔE (a.u.)	ΔE (kJ mol^{-1})
E3	−908.9173	–	–
EE	−871.2266	–	–
BPA	−718.0582	–	–
Phe	−301.7330	–	–
MAA	−300.7518	–	–
EE: 2MAA	−1472.7480	0.0178	46.7
E3: 2MAA	−1510.4371	0.0162	42.5
BPA: 2MAA	−1319.5842	0.0244	58.8
Phe: MAA	−602.4942	0.0094	24.7

rebinding capacities of EE and E3 in E2-MMIPs were lower than that of E2 due to the ethynyl group in position 17 of EE and the hydroxyl group in position 16 of E3, which could result in steric hindrance when they bind with the functional group at the specific binding sites. On the contrary, the binding capacity of BPA and Phe was low, and the main reason might be the mismatch of their structure and size with the specific cavities on the surface of E2-MMIPs. So the more similar structure of compound is, the higher the binding capacity is.

3.4. Optimization of the clean-up and elution procedure

The types and volume of extracting and eluting solvent are the key factors, which should be carefully selected to achieve the highest recovery for analytes while eliminating most of the interferences originated from complicated matrix. Different extracting solvents such as methanol, acetonitrile and toluene–methanol (9:1, v/v) and the volume of solvent ranged from 0.25 mL to 1.0 mL were investigated and better recoveries and shortest extracting time were obtained when 0.5 mL toluene–methanol (9:1, v/v) were used as the extracting solvents.

Different elution solvents such as methanol and 1 M HCl (1:1, v/v), methanol-acetic acid (9:1, v/v), acetonitrile–acetic acid (9:1, v/v), and acetonitrile–trifluoracetic acid (99.5:0.5, v/v) were investigated and the best recoveries were obtained by using methanol and 1 M HCl (1:1, v/v) as elution solutions. On the other hand, the volumes of solvent ranged from 0.25 mL to 1.0 mL were also investigated and 0.5 mL of the solvent was found to be the optimum volume and the recoveries of the E2 in this case were all above 90.0%.

3.5. Method validation and application to feed samples

Under the optimized conditions of HPLC–UV coupled with E2-MMIPs-DSPE procedure, good linearity was achieved in range of 0.1–4 μM for E2 and E3 with correlation coefficient of 0.9996 and 0.9994, respectively. The LOD was 0.03 μM of E2 and E3, respectively.

The accuracy of the method was estimated by determining three different kinds of feeds (chicken, goose or fish feeds) samples spiked with E2 and E3 at three different concentration levels. As could be seen in Table 3, the recoveries of E2 with the E2-MMIPs-DSPE for chicken, goose and fish feeds were in the range 92.7–97.0% with RSD < 4.7%, and the recoveries of E3 were in the range 71.9–83.1% with RSD < 4.9%. The results demonstrated that E2-MMIPs had good recovery and reproducibility even at the low concentration, and could satisfy the determination of E2 and E3 in the three different kinds of feed samples.

Table 3
Determination of estrogens in three feeds ($n = 5$).

Sample	Determined level (mg kg^{-1})		Spiked level (mg kg^{-1})		Recovery (%)		RSD (%)	
	E2	E3	E2	E3	E2	E3	E2	E3
Chicken feed	3.06	1.72	1.50	0.87	96.5	72.3	3.4	4.1
			3.00	1.74	96.1	83.1	2.8	2.9
			4.50	2.61	94.7	77.8	1.3	3.6
Goose feed	2.39	0.52	1.20	0.25	96.2	79.6	4.3	4.8
			2.40	0.50	95.3	75.4	4.6	3.7
			3.60	0.75	93.4	71.9	3.8	2.2
Fish feed	2.68	0.69	1.36	0.35	97.0	78.5	2.7	4.2
			2.72	0.70	96.6	74.2	1.4	3.7
			4.08	1.05	92.7	80.7	2.9	3.1

Fig. 5A shows the chromatograms of E2 and E3 in three feeds, which were obtained with direct injection (picture a), E2-MMIPs-DSPE (picture b) and MNIPs-DSPE (picture c), respectively. The targets in the obtained solution with E2-MMIPs-DSPE were E2 and E3 by LC–MS/MS, respectively (Fig. 5B).

As could be observed from the chromatograms, the concentration of E2 and E3 was high enough to be quantitatively analyzed and most interfering compounds were removed after E2-MMIPs-DSPE procedure comparing with direct injection and MNIPs-DSPE procedure. Furthermore, due to the selective binding sites at the surface of E2-MMIPs, the DSPE procedure can reach the equilibrium quickly, and does not require special instrumentation, consumes much less toxic organic, and has a good clean-up and concentration effect for the analytes. These characteristics proved that E2-MMIPs-DSPE coupled with HPLC was a suitable method for selective extraction and sensitive determination of E2 and E3 in agricultural products.

4. Conclusions

In this paper, a novel, efficient and nano-sized E2-imprinted sorbent was synthesized by a surface molecular self-assembly strategy and most of recognition sites of E2-MMIPs were produced at surface or in the proximity of the surface, so the diffusion resistance to bring the analytes into the recognition sites could be decreased. E2-MMIPs showed that sufficient magnetite was encapsulated and the magnetic separation process could be performed directly in complex samples. In the adsorbing experiment, high selectivity, good reproducibility, large adsorption capacity and fast binding kinetics for E2 were displayed. Meanwhile, the combination of E2-MMIPs-DSPE and HPLC–UV was developed to determine the trace amounts of estrogens in feed samples. The approach described here will open a new window in the applications involved in the quick separation, high selectivity, fast enrichment and straightforward extraction of estrogens from complicated matrices.

Acknowledgments

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