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Analytical Methods

Magnetic nanoparticles based dispersive micro-solid-phase extraction as a novel technique for the determination of estrogens in pork samples

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ABSTRACT

A simple and rapid magnetic nanoparticles (MNPs) based dispersive micro-solid-phase extraction (D-μ-SPE) method coupled with HPLC-DAD has been proposed for simultaneous determination of three estrogens (17β-estradiol (E2), estrone (E1) and diethylstilbestrol (DES)) in pork samples. In this paper, the synthesis of cetyltrimethyl ammonium bromide (CTAB)-coated Fe_3O_4@caprylic acid NPs as an efficient sorbent for its high surface area, excellent adsorption capacity, good dispersion ability and high superparamagnetic property was successfully applied to adsorb estrogens. Vortex was used to enhance mass transfer rate as it provided mild and effective mixing of sample solution and increased the contact between analytes and MNPs. The parameters affecting the extraction efficiency were investigated in detail. The dosages of sorbent and eluate are 100 μL and 500 μL, respectively. The extraction equilibrium was achieved within 2 min and the MNPs can be reused. The proposed technique provided high recoveries (93.3–106.7%), good linearity (0.9993–0.9999), low LODs (0.021–0.033 ng mL^{-1}) and repeatability (RSD% = 1.87–2.92).

1. Introduction

Steroid estrogens are a large group of lipophilic, low-molecular weight, high estrogenic active compounds, and they were roughly classified as natural and synthetic estrogens (Wang et al., 2011). Natural estrogens (also called endogenous estrogens) include estradiol (E2) and its most common metabolites or precursors: estrone (E1) and estriol (E3). These estrogens can promote growth of animals and improve the conversion efficiency of feeds, so they were extensively used in animal husbandry (Lammers, Heinrichs, & Kensinger, 1999). Also, it has been reported that estrogens existed in aquatic environments (Hecker, Tyler, Hoffmann, Maddix, & Karbe, 2002; Johnson, & Sumpter, 2001) and caused the feminization of male fish at much lower concentrations (1 pg mL^{-1}) in aquatic environment (Hansen et al., 1998). In consideration of the possible harmful effects on public health (Fuh, Huang, & Lin, 2004), the use of estrogens in food producing animals has been prohibited in European Community and China (Xu et al., 2013). 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 samples at low concentration level (Wang et al., 2011). Therefore, developing a selective, accurate and sensitive analytical method for detecting estrogens in meat samples is crucial for the investigation of potential use of estrogens in food-safety area.

Up to now, several analytical methods have been described for estrogens analysis including high performance liquid chromatography (HPLC–DAD), liquid chromatography with mass spectrometry (LC-MS) (Iparraguirre et al., 2014), gas chromatography (GC) and gas chromatography with mass spectrometry (GC–MS) (Hansen et al., 2011), as well as micellar electrokinetic chromatography (MEKC) (Wen et al., 2013). These chromatographic methods involve traditional sample pretreatment procedures, such as liquid–liquid extraction (LLE) (Fernandez, Ikonomou, & Buchanan, 2007), liquid phase microextraction (LPME) and dynamic liquid–liquid–solid microextraction (DLLSME) (Zhong, Hu, Hu, & Li, 2012), ultrasound-assisted surfactant-enhanced emulsification microextraction (UASEME) (Zou et al., 2012), solid phase extraction (SPE) (Zhang, You, Ning, Song, & Suo, 2013), solid phase micoextraction (SPME) (Lan et al., 2014), stir bar sorptive extraction (SBSE) (Hu, Zheng, Zhu, & Li, 2007) and micro-solid-phase extraction (μ-SPE) (Wang et al., 2013).

SPE is one of the most popular sample pretreatment methods and has many significant advantages, such as improvements in

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automation, reproducibility and high-throughput capability (Li, & Lee, 2001; Mitani, Fujioka, & Kataoka, 2005). Dispersive micro-solid phase extraction (D-μ-SPE), which is categorized as a SPE technique, has many advantages compared to the traditional SPE such as short time requirement and reduced solvent consumption (Basheer, Alnedhary, Rao, & Lee, 2009; Basheer, Chong, Hii, & Lee, 2007), economic and easy to perform and convenience for efficiency of recovery. Magnetic carrier technology (MCT), based on magnetic nanoparticles (MNPs) for their high surface area-to-volume ratio and super-paramagnetism property, was first reported by Robinson (Robinson, Dunnill, & Lilly, 1973) and became popular as an analysis tool in analysis area. Naked Fe_3O_4 NPs, which have large surface area, were easy to cause particles agglomeration and form large clusters. Therefore, it is necessary to engineer the surface of MNPs to minimize their agglomeration problem through coating/modifying processes (Beiraghi, Pourghazi, & Amoli-Diva, 2014). Besides, the sensitivity and selectivity of MNPs were obtained by the modification of surface with functionalities (Moller, Kobler, & Bein, 2007; Tahmasebi, Yamini, Seidib, & Rezazadeh, 2013).

The purpose of this work is to develop a MNPs based D-μ-SPE method combining with HPLC–DAD for the preconcentration and determination of estrogens in pork samples. CTAB-coated Fe_3O_4@caprylic acid was used as sorbent and vortex was utilized as an assisted approach to accelerate the mass transfer. In addition, the sorbent was separated from the aqueous samples by an external magnet. E2, E1, and DES were selected as model compounds for examine the feasibility of method. Affecting factors of the CTAB-coated Fe_3O_4@caprylic acid based D-μ-SPE of three target estrogens (sorbent type, sorbent dosage, CTAB amount, sample pH, extraction time, and salt concentration) were investigated and optimized and the proposed method was successfully applied to the extraction and preconcentration of estrogens in pork samples.

2. Experimental

2.1. Chemicals and materials

Standards of 17β-estradiol (E2, 99.5%), estrone (E1, 99.0%), and diethylstilbestrol (DES, 99.7%) were supplied by Sigma (Sigma, USA). Ferric chloride ($FeCl_3$), ammonium ferrosulfate ($(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$), ammonium hydroxide (28%, w/v), hexanoic acid (HA), caprylic acid (CA), decanoic acid (DA) and cetyltrimethyl ammonium bromide (CTAB) were all purchased from Aladdin Chemistry (Shanghai, China). Methanol and acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Ultra-pure water was produced by a milli-Q system (Bedford, MA, USA). All reagents were at or above the analytical reagent grade.

2.2. Apparatus

An Agilent 1200 Series HPLC system (Agilent Technologies, Calif., USA) was used for Chromatographic separation and evaluation. This HPLC system includes an auto sampler, vacuum degasser, quatpump, and diode array detector, which is equipped with a reversed-phase C18 analytical column of 150 × 4.6 mm (Agilent TC-C18). Empowered software was employed to acquire and analyze chromatographic data.

Fourier transform infrared spectra (FTIR) 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 were used in the procedure, including a CS-400 transmission electron microscopy (TEM) (No. 45 Research Institute of CETC, China), a D8-advance X-ray diffraction (XRD) (Bruker, Germany), a sample bead homogeneous instrument (Pingli foreign trade and economic Ltd., Beijing, China), a vortex agitator (Kylin-Bell Lab Instruments Co. Ltd., Jiangsu, China), an ultrasonic cleaner (Kunshan ultra-sonic instrument plant, Jiangsu, China), a water bath (Shanghai happy instrument equipment Co. Ltd., Shanghai, China), a mechanical stirrer (Huanglong experiment instrument plant, Jiangsu, China) and a vacuum oven (Shanghai Yuezhong instrument equipment Co. Ltd., Shanghai, China). A strong Nd-Fe-B magnet (New magnetic factory, Guangzhou, China) was used for sorbent collection and magnetic decantation.

2.3. Chromatographic conditions

Acetonitrile and water were used as mobile phases and the gradient program contained the following steps: linear gradient of 35% acetonitrile for 0–4.5 min, from 35% to 55% acetonitrile for 6.0–14 min, from 55% to 35% acetonitrile for 14–16 min. The flow rate was set at 1.0 mL min^{-1}, the injection volume was 10 μL and the column temperature was maintained at 25 °C. The detection wavelength was at 280 nm.

2.4. Preparation of standard solutions and real samples

Stock standard solution of estrogens (1000 μg mL^{-1}) was prepared in methanol and stored in dark at 4 °C, which can be used for 2 months. Standard working solutions were prepared by dilution of the stock solution in methanol before use.

The fresh pork samples were purchased from supermarkets in Kunming, China. These pork samples were stored at 4 °C until analysis. A piece of fresh pork sample (about 2.0 g) was crushed to homogenate by using a sample bead homogeneous instrument, followed by adding 2 mL of methanol and sonicating for 20 min at room temperature. The organic phase was diluted to 5 mL with ultra-pure water for MNPs based D-μ-SPE procedure.

2.5. Synthesis of the MNPs

2.5.1. Synthesis of Fe_3O_4@caprylic acid NPs

Analogously to the synthesis method of Fe_3O_4@caprylic acid NPs which was studied by Asgharinezhad, Mollazadeh, Ebrahimzadeh, Mirbabaei, & Shekari (2014). Briefly, 2.05 g of ammonium ferrosulfate and 1.41 g of ferric chloride were dissolved under N_2 atmosphere in 40 mL of ultra-pure water with constant stirring at 1500 rpm using a mechanical stirrer. 6 mL of $NH_3{\cdot}H_2O$ and 2 mL of caprylic acid were added when the solution was heated to 80 °C. The crystal growth was allowed to proceed in water bath at 80 °C for 30 min under vigorous stirring under N_2 atmosphere. Then the water-based suspension was cooled down to room temperature slowly. After that the suspension was precipitated with ethanol and ultra-pure water, and the precipitates were isolated from the supernatant solution by magnetic decantation. This washing decantation procedure was repeated six times to remove the excess of caprylic acid. At last, the obtained Fe_3O_4@caprylic acid NPs were vacuum-dried at 55 °C for 10 h. Then the synthesized Fe_3O_4@caprylic acid NPs were stored at 4 °C.

2.5.2. Synthesis of other functionalized NPs

The synthesis of Fe_3O_4@HA NPs and Fe_3O_4@DA NPs was similar with the above synthesis of Fe_3O_4@caprylic acid NPs.

The synthesis of Fe_3O_4 NPs: 2.05 g of ammonium ferrosulfate and 1.41 g of ferric chloride were dissolved under N_2 atmosphere in 40 mL of ultrapure water with constant stirring at 1500 rpm using a mechanical stirrer. 6 mL of $NH_3{\cdot}H_2O$ was added when the solution was heated to 80 °C. The crystal growth was allowed to proceed in water bath at 80 °C for 30 min under vigorous stirring under N_2 atmosphere. Then the water-based suspension was cooled down to room temperature slowly. After that the

suspension was precipitated with ethanol and ultra-pure water, and the precipitates were isolated from the supernatant solution by magnetic decantation. This washing decantation procedure was repeated four times. At last, the obtained Fe_3O_4 NPs were vacuum-dried at 55 °C for 10 h. Then the synthesized Fe_3O_4 NPs were stored at 4 °C.

2.6. MNPs based D-μ-SPE procedure

The extraction procedure was accomplished as the follows:

(1) Preparation of MNPs suspension
600 mg of dried Fe_3O_4@caprylic acid NPs and 0.05 mL of CTAB (6.0 mg mL^{-1}) were added in 10 mL of ultra-pure water and sonicated for 10 min to make MNPs suspension disperse well-distributed.

(2) Extraction of the experiment
0.10 mL of above MNPs suspension was added to 5 mL sample solution containing 50 ng mL^{-1} of each estrogen. The mixture was vortex-mixed for 1 min to make estrogens full adsorbed by the MNPs. Then, MNPs were separated quickly from the sample solution by using an external magnet, the supernatant was decantated and the MNPs were eluted by 500 μL of methanol. Finally, the eluate was isolated from MNPs by using an external magnet and filtered by a 0.45 μm membrane and injected into the HPLC instrument for subsequent analysis.

3. Results and discussion

3.1. Physical characteristics of Fe_3O_4@CA NPs

TEM technique was used to explore the morphology and dimension of Fe_3O_4 NPs and Fe_3O_4@CA NPs. As shown in Fig. 1A, Fe_3O_4 NPs and Fe_3O_4@CA NPs are nearly spherical in shape with an average diameter of 10–20 nm. In addition, they tended to aggregate to large particles due to their large specific surface area, high surface energy and magnetization.

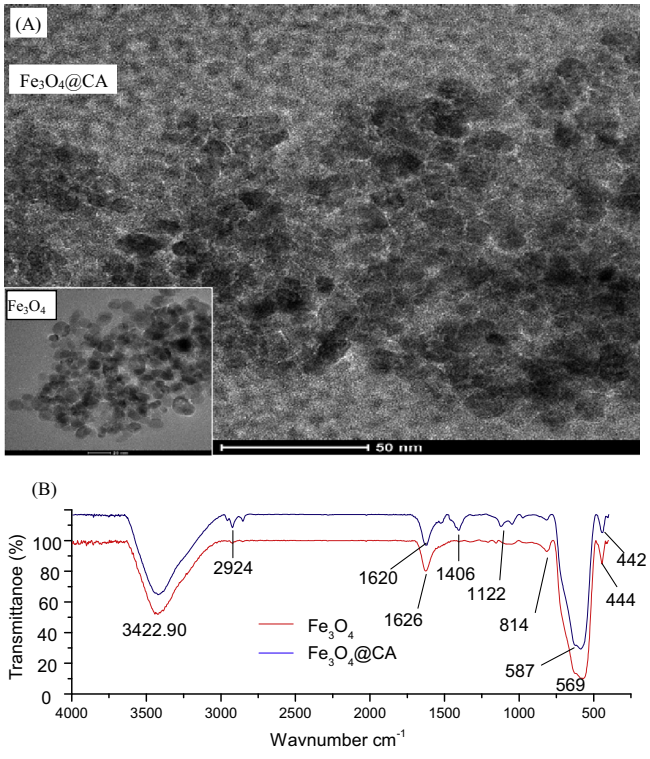

Fig. 1. TEM image (A) and FTIR spectra (B) of the Fe_3O_4 and CTAB-coated Fe_3O_4@CA NPs.

The surface chemistry of Fe_3O_4 and Fe_3O_4@CA NPs was studied using FTIR spectrum. The typical FTIR spectra of Fe_3O_4 and Fe_3O_4@CA NPs were shown in Fig. 1B. As can be seen, an absorption band appeared at 569.43 cm^{-1} corresponding to the Fe–O bond in the Fe_3O_4 particles. Compared with Curve (Fe_3O_4), Curve (Fe_3O_4@CA) displayed clearly the characteristic peaks of Fe_3O_4@CA NPs including methyl (–CH_3) and methylene (–CH_2–) at approximately 2923 cm^{-1} and 1620 cm^{-1} (Lan et al., 2014), carbonyl (C=O) stretching vibration at 1406.40 cm^{-1} (Yuan et al., 2011), which all confirmed the generation of the Fe_3O_4@CA NPs.

The X-ray power diffraction (XRD) patterns were tested for the synthesized Fe_3O_4 and Fe_3O_4@CA NPs. Six characteristic peaks for Fe_3O_4 were observed in Fe_3O_4 and the peak positions at the corresponding 2 value were indexed as (220), (311), (400), (422), (511) and (440), respectively. Also, Fe_3O_4 and Fe_3O_4@CA NPs had similar diffraction peaks, which indicated that the crystal structure of Fe_3O_4 NPs was not changed after modification with CA.

3.2. Optimization of MNPs based D-μ-SPE parameters

3.2.1. Sorbent type

In this context, extraction abilities of Fe_3O_4 NPs, CTAB-coated Fe_3O_4 NPs, Fe_3O_4@HA NPs, Fe_3O_4@CA NPs, Fe_3O_4@DA and CTAB-coated Fe_3O_4@CA NPs were investigated as the sorbent type was an important parameter affecting the extraction efficiency. The results indicated that CTAB-coated Fe_3O_4@CA NPs can act as the best sorbent on account of the presence of caprylic acid and CTAB on the surfaces of Fe_3O_4 NPs. Compared to hexanoic acid and decanoic acid, caprylic acid has a good match with analytes. Moreover, Fe_3O_4@CA NPs could disperse evenly in aqueous solution by adding CTAB. Compared to bare Fe_3O_4 NPs, hydrophobic interactions and π–cation between analytes and Fe_3O_4 have been enhanced at the existence of caprylic acid and CTAB on the surfaces of NPs. The estrogens can interact through hydrophobic interaction with carbon chain of caprylic acid and CTAB. What's more, π–cation interactions between the quaternary ammonium group of CTAB and the aromatic rings in the analytes are possible. Therefore, CTAB-coated Fe_3O_4@CA NPs were selected for the rest of experiments.

3.2.2. Effect of sorbent dosage and reuse time

In order to select the optimum amount of sorbent for the extraction of estrogens, different dosages of Fe_3O_4@caprylic acid NPs (60 mg mL^{-1}) were tested across the range 50–175 μL. The results indicated that satisfactory recoveries for the extraction of analytes could be obtained using 100 μL of Fe_3O_4@caprylic acid NPs under the same conditions. Nano-sized sorbents have greater surface areas than ordinary sorbents and satisfactory results can be obtained by lower dosages of MNPs. When beyond 100 μL, all the MNPs cannot be separated effectively and lead to a slightly decrease in recoveries. Therefore, 100 μL of Fe_3O_4@caprylic acid NPs was used in the next experiments.

The reuse time of CTAB-coated Fe_3O_4@caprylic acid NPs was investigated in the experiment. The recoveries were above 90% as CTAB-coated Fe_3O_4@caprylic acid NPs were used at 1–3 times. With the increase of reuse time, the recoveries gradually reduced. Hence, the CTAB-coated Fe_3O_4@caprylic acid NPs were reused for three times.

3.2.3. Effect of sample solution pH

Generally, the pH of the sample solution plays an important role in adsorption process as it determines the form of analytes in aqueous solution and the surface charge of sorbent (Asgharinezhad

et al., 2014). The influence of pH of the sample on extraction efficiency was studied over the pH in the range of 4–9. The surface charge of Fe_3O_4 NPs is neutral at pH_{pzc} (the pH value is at the point of zero charge), which is about 6.5 (Zhao, Shi, Wang, Cai, & Jiang, 2008). While in the presence of caprylic acid, pH_{pzc} is different from the bare Fe_3O_4 NPs and is lower than 6.5. Beyond the pH_{pzc}, the estrogens cannot directly interact with sorbent surface due to the surface charge of sorbent and estrogens become negative. While below the pH_{pzc}, the surface charge of the sorbent (Fe_3O_4@caprylic acid NPs) is positive and the estrogens are neutral. The estrogens can interact through hydrophobic interaction with carbon chain of caprylic acid CTAB and CTAB. Moreover, π–cation interactions between the quaternary ammonium group of CTAB and the aromatic rings in the target analytes are other possible interactions. Hence, pH 5.0 was used in the subsequent experiments.

3.2.4. Effect of CTAB dosage

CTAB plays a prominent role in the extraction mechanism of estrogens and affects the adsorption efficiency of estrogens. Therefore, the CTAB (8.0 mg mL^{-1}) dosages of 20, 40, 60, 80, 100 μL were tested to evaluate the effect of CTAB. In the optimum pH, the estrogens are adsorbed through hydrophobic chain of CTAB and caprylic acid on the surfaces of MNPs. Moreover, the solubility of Fe_3O_4@caprylic acid NPs increased with the increase of CTAB. However, at high concentrations of CTAB, Fe_3O_4@caprylic acid NPs cannot completely be separated from the sample solution by using a magnet. Hence, the amount of 60 μL of CTAB was selected as optimum value for the subsequent experiments. The dispersity and magnetic separability of CTAB-coated Fe_3O_4@CA NPs suspension (60 mg mL^{-1}) were investigated in Fig. 2.

3.2.5. Effect of vortex extraction time

The extraction of analytes can be achieved in a shorter time as the shorter diffusion route for NPs and the magnetically assisted separation of the MNPs from the sample solutions and vortex-assisted method was developed. To evaluate the effect of vortex extraction time on the extraction efficiency of the estrogens, the extraction times ranged from 0.5 to 3 min were varied. High recoveries were got with vortex for 2 min. Therefore, 2 min was selected as vortex extraction time.

3.3. Validation of the proposed MNPs based D-μ-SPE method

To evaluate the proposed MNPs based D-μ-SPE method, regression equations, correlation coefficient (r), linearity range, limits of detection (LODs) and limits of quantitation (LOQs) were investigated under the optimum conditions. The linearity range was assessed by plotting the peak area against the concentration of the respective compounds for quantitative purposes. The correlation coefficients (r) values were 0.9998, 0.9993, and 0.9999 for E2, E1 and DES, respectively and good linearity in the range of 5–1000 ng mL^{-1} was observed. The LODs based on signal-to-noise ratio of 3 were 0.033 ng mL^{-1} for E2, 0.031 ng mL^{-1} for E1, and 0.021 ng mL^{-1} for DES, respectively. The LOQs based on signal-to-noise ratio of 10 were 0.111 ng mL^{-1} for E2, 0.112 ng mL^{-1} for E1, and 0.071 ng mL^{-1} for DES, respectively. Moreover, the relative standard deviations (RSDs%) in quintuple of 50 ng mL^{-1} for E2, E1, and DES were 1.87, 2.02, 1.99, respectively.

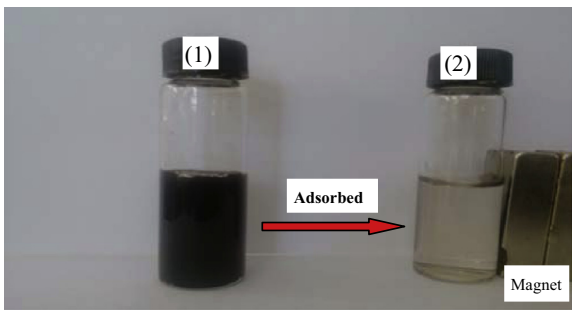

Fig. 2. Photographs of MNPs based D-μ-SPE steps: (1) extraction, (2) magnetic separation. Sorbent: CTAB-coated Fe_3O_4@CA NPs.

3.4. Analysis of pork samples

To evaluate the accuracy and applicability of the proposed method for the determination of estrogens in pork samples, two kinds of pork samples were used in the experiments. 1.15 mg/kg of E2 and 1.24 mg/kg of DES were detected in pork samples. The detail data were shown in Table 1. Under the optimized conditions, pork samples were spiked at 1, 10, and 100 mg/kg levels, and the mean recoveries were in the range of 93.3–106.7%, with the relative standard deviations (RSDs) were in the range of 1.87–2.92%. The typical HPLC chromatograms of estrogens adsorbed from pork samples were shown in Fig. 3.

3.5. Comparison of the proposed method with other reported methods

Table 2 compares the figures of merit of the proposed MNPs based D-μ-SPE method and other reported pretreatment techniques for the extraction of E2, E1 and DES in blood, urine, milk, milk power, and meat samples. As shown in Table 2, the proposed method has high recoveries and low LODs. Besides, this method required only a very short extraction time, with a small amount of organic solvent. All of these were indicated the analytical performance of the proposed MNPs based D-μ-SPE method is feasibility.

Table 1
Determination of estrogens in pork samples (n = 2).

Analyte	Sample	Spiked (mg/kg)	Found (mg/kg)	Recovery (%)	RSD (%, n = 5)
E2	Pork sample 1	0	1.15	–	–
		1	2.11	96.0	1.87
		10	10.51	93.6	2.53
		100	98.05	96.9	2.92
	Pork sample 2	0	nd[a]	–	–
		1	0.97	97.0	2.74
		10	9.33	93.3	1.98
		100	98.9	98.9	2.35
E1	Pork sample 1	0	nd[a]	–	–
		1	0.94	94.0	2.47
		10	9.58	95.8	2.58
		100	93.9	93.9	2.87
	Pork sample 2	0	nd[a]	–	–
		1	0.95	95.0	2.82
		10	95.9	95.9	2.90
		100	94.7	94.7	2.75
DES	Pork sample 1	0	1.24	–	–
		1	0.98	98.0	2.79
		10	11.03	97.9	2.87
		100	106.44	105.2	2.69
	Pork sample 2	0	nd[a]	–	–
		1	0.99	99.0	1.91
		10	10.67	106.7	2.89
		100	100.4	100.4	2.34

[a] Not detected.

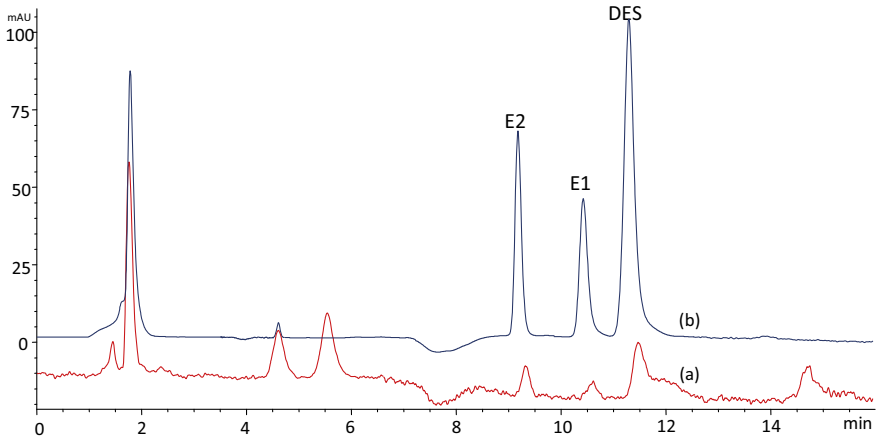

Fig. 3. Typical HPLC/DAD chromatograms: 2.0 g of pork sample spiked with 50 ng mL^{-1} estrogens without D-μ-SPE (a) and by CTAB-coated Fe3O4@CA NPs based D-μ-SPE (b).

Table 2
Comparisons of the proposed method with other sample preparation techniques for the determination of estrogens.

Sample	Analytes	Sample preparation	Solvent amount	Extraction time (min)	Recovery (%)	RSD (%)	LODs (ng/mL)	References
Milk	E2, EE, E1, DES, DHDS	DLLSME	200 μL	30	81.9–99.8	0.11–2.68	0.32–93.8	Zhong et al. (2012)
Urine	βE2, E1, DES	UASEME	>500 μL	5	89.8–104.4	4.1–7.9	0.08–0.25	Zou et al. (2012)
Meat	E1, E2, DES, BPA, E3, OP, NP	SPE	>1 mL	>5	75.3–92.5	3.2–5.2	0.05–0.17	Zhang et al. (2013)
Milk power	E1, E2, E3, DES	SPME	2–10 mL	20	81.5–96.6	4.0–6.1	1.5–5.5	Lan et al. (2014)
Blood	E1, DES, BPA, DE, 4-NP, 4-tOP, HEX, DE	D-μ-SPE	1 mL	1	85–105	4.9–5.2	0.022–0.14	Zhao et al. (2013)
Meat	E1, E2, DES	MNPs based D-μ-SPE	500 μL	2	93.3–106.7	1.87–2.92	0.021–0.033	This work

4. Conclusions

In this study, the CTAB-coated Fe_3O_4@caprylic acid NPs based D-μ-SPE method has been developed for preconcentration and determination of E2, E1 and DES in pork samples. Fe_3O_4 NPs were modified with caprylic acid and CTAB to minimize their agglomeration problem and maximize the selective adsorption of estrogens. The extraction equilibrium was achieved in 2 min and the synthesized MNPs with diameters of 10–20 nm can be reused for 3 times. Besides, the proposed method has other advantages such as convenience for operation, and reduced solvent consumption in compare with the conventional SPE methods. High recoveries, low detection limits and wide linearity range were obtained and indicated CTAB-coated Fe_3O_4@caprylic acid NPs based D-μ-SPE method coupled with HPLC has high analytical potential for the preconcentration and determination of E2, E1 and DES in pork samples or other complex samples.

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