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Rapid Determination of Estrogens in Milk Samples Based on Magnetite Nanoparticles/Polypyrrole Magnetic Solid-Phase Extraction Coupled with Liquid Chromatography−Tandem Mass Spectrometry

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ABSTRACT: In this study, a nanocomposite of polypyrrole-coated magnetite nanoparticles (denoted as MNPs/PPy) was prepared and employed as magnetic solid-phase extraction (MSPE) sorbent for extraction of estrogens from milk samples. Because the polypyrrole coating possessed a highly π-conjugated structure and hydrophobicity, MNPs/PPy showed excellent performance for the estrogen extraction. Estrogens could be captured directly by MNPs/PPy from milk samples without protein precipitation. Moreover, the extraction could be carried out within 3 min. Thus, a rapid, simple, and effective method for the analysis of estrogens in milk samples was established by coupling MNPs/PPy-based MSPE with liquid chromatography−tandem mass spectrometry (LC−MS/MS). The limits of detections for estrogens investigated were in the range of 5.1−66.7 ng/L. The recoveries of estrogens (concentration range of 0.5−20 ng/mL) from milk samples were in the range of 83.4−108.5%, with relative standard deviations ranging between 4.2 and 15.4%.

KEYWORDS: Estrogens, magnetite nanoparticles/polypyrrole, magnetic solid-phase extraction, LC−MS/MS

INTRODUCTION

Estrogens, a group of steroid hormones, have attracted increasing attention from the scientific community, governments, and the general public.[1] It has been confirmed that the increased incidence of male reproductive disorders, such as testicular cancer and poor semen quality, is related to the increased levels of circulating estrogens in the body.[2] Recently, it has been found that there is a strong correlation between estrogens and human carcinogenesis, such as breast, prostate, and ovarian cancers.[3,4] There are therefore substantial scientific efforts in progress to assess the levels of these compounds in various samples.[5,6] However, most researches focus on the presence of estrogens in environmental water and surface sediment matrices because they are considered to be the rich sources of estrogens; only a few studies have been devoted to the determination of estrogens in milk matrices.[7−9] Actually, milk occupies a very important position in the human diet structure.[10−12] It is reported that 75% of milk is produced from pregnant cows in the modern dairy industry; thus, the content of estrogens in commercial milk shows a sustained rise.[8] Therefore, it is of significance to develop rapid, simple, and effective methods for the analysis of estrogens in milk samples.

Generally, the estrogens in milk are in low concentration and simultaneously accompanied by a large number of interfering substances. Thus, sample enrichment and purification techniques are highly required prior to chromatography and/or mass spectrometry analysis. Among various available methods, the solid-phase extraction (SPE) technique is particularly attractive because of its numerous advantages, such as simplicity, flexible selection of sorbents, and low consumption of the organic solvents.[13−15] Recently, some attempts have been made to develop SPE methods for extracting estrogens from milk matrices.[7−9] However, all of these reported SPE processes needed the time-costing step of column passing and filtration operation because the adsorbent materials were invariably packed into cartridges. Thus, novel SPE modes that can facilitate mass transfer are desirable.

Magnetic solid-phase extraction (MSPE) is a new mode of SPE based on the use of magnetic or magnetizable adsorbents, and MSPE shows great advantages in separation science now.[16−20] The adsorbent does not need to be packed into the SPE cartridge; instead, it can be dispersed in a sample solution or suspension. The powdery magnetic adsorbent can be reversibly agglomerated and redispersed in solution or suspensions by the application and removal of an appropriate magnetic field; thus, the phase separation could be conveniently conducted. From the view of mass transfer, the MSPE mode can also facilitate mass transfer of analytes by drastically increasing the interfacial area between the solid adsorbent and sample solution.[18] However, to the best

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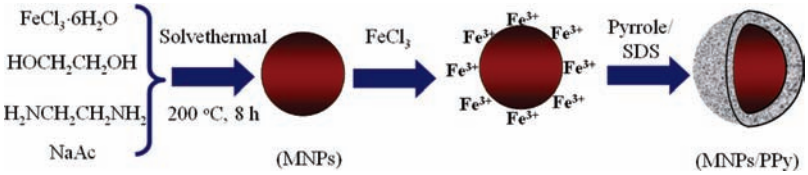

Figure 1. Preparation scheme of MNPs/PPy.

of our knowledge, until now MSPE has not been applied to estrogen extraction from milk samples. On the other hand, polypyrrole (PPy), one of the most important conducting polymers, has been studied extensively in its great potential application in many fields.[21−23] Previous studies demonstrated that PPy was an efficient SPE material for the extraction of aromatic compounds [e.g., polycyclic aromatic hydrocarbons (PAHs)] and other organic compounds from a series of matrices[17,24−28] because of the formation of the $\pi-\pi$ complex and/or hydrophobic interactions between PPy and analytes.

In this study, we synthesized a nanocomposite of PPy-coated magnetite nanoparticles (i.e., MNPs/PPy), and the resultant material was demonstrated to be able to efficiently capture estrogens from milk samples. Interestingly, MNPs/PPy could directly extract estrogens from milk samples without protein precipitation. Coupled with liquid chromatography−tandem mass spectrometry (LC−MS/MS), a rapid, simple, and effective MSPE−LC−MS/MS method for the analysis of estrogens in milk samples was established.

MATERIALS AND METHODS

Reagents and Chemicals. Pyrrole, sodium dodecyl sulfate (SDS), ethylene glycol (EG), ethanol, ethylene diamine (ED), ferric trichloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferric chloride ($FeCl_3$), and sodium acetate (NaAc) were purchased from Sinopharm Chemical Reagent (Shanghai, China). All of these chemicals were used directly without further purification.

Seven estrogens, namely, estrone (E_1, 99.0%), β-estradiol (E_2, 99.0%), estriol (E_3, 97.0%), dienestrol (DS, 99.5%), hexestrol (HEX, 98.5%), diethylstilbestrol (DES, 99.0%), and ethinylestradiol (EE_2), were purchased from the Laboratories of Dr. Ehrenstorfer (Augsburg, Germany). A mixed stock solution of the estrogens was prepared in methanol at a concentration of 1 mg/mL and stored at 4 °C in darkness. Preliminary experiment results showed that the stock solutions were stable for almost 3 months. With the stock solutions, the sample solution was spiked to the desired concentration for the following experiments.

High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol was purchased from Fisher Company, Inc. (Fair Lawn, NJ). Acetone was obtained from J. T. Baker Chemical Company (HPLC grade, Phillipsburg, NJ). Purified water was obtained with a Millipore Milli-Q apparatus (Bedford, MA).

Milk Samples. All milk samples were purchased from local markets in Wuhan (China) and stored at −20 °C. One milk sample was checked to be free of any of the selected estrogens and used as blank milk for calibration and validation purposes. The seven estrogens were directly spiked into 1 mL of milk sample over a range of 0.08−40 ng/mL. After mixing evenly, the sample was diluted to 10 mL with phosphate buffer (10 mM, pH 7.0) before use. Blank milk samples were prepared in the same way as described above but without the analyte-spiking step.

Preparation of PPy/MNPs. The preparation procedure of PPy/MNPs is depicted in Figure 1, including the following two steps.

First, MNPs were synthesized via a solvothermal process according to our previously used method.[20] Briefly, $FeCl_3 \cdot 6H_2O$ (5.0 g) was dissolved in EG (100 mL), and then NaAc (15.0 g) and ED (50 mL) were added to the solution. After vigorous stirring for 30 min, the homogeneous mixture was sealed in a Teflon-lined stainless-steel autoclave (200 mL). The autoclave was heated to 200 °C, maintained for 8 h, and allowed to cool to room temperature. The product was magnetically collected, washed with water/ethanol several times, and vacuum-dried at 60 °C for 6 h.

Then, the resultant MNPs were PPy-coated according to the method described by Luo et al. with some modifications.[29] Typically, 1.0 g of MNPs, 9.1 g of $FeCl_3$, and 100 mL of deioned water were added to a 250 mL flask. The mixture was continuously shaken with a speed of 150 shakes/min in a water bath at 25 °C for 3 h to accumulate the Fe^{3+} ions on the surface of MNPs by the common ion effect.[29] Then, 20 mL of SDS solution (5.85 wt %) and 0.5 mL of PPy monomers were rapidly added, and the mixture was kept shaking for 12 h. The products (i.e., MNPs/PPy) were magnetically collected, washed by water/ethanol successively and repeatedly, and then vacuum-dried at 60 °C for 6 h.

The MNPs and MNPs/PPy were characterized by a JEM-100CXII transmission electron microscope (TEM, Jeol, Japan), AVATAR 360 FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA), and PPMS-9 vibrating sample magnetometer (Quantom, San Jose, CA).

MSPE Procedure. MNPs/PPy (5 mg) were put into a 15 mL vial and first activated with methanol and Milli-Q water in sequence, and then 10 mL of diluted milk was added to the vial. After vortexing for 3 min, the estrogen-adsorbed MNPs/PPy were magnetically separated from the milk solution with the assistance of a magnet and then washed with 2 mL of water. After the washing solution was discarded, estrogens were desorbed with 1 mL of acetone under vortex (0.5 min). The solution was separated from MNPs/PPy by a magnet and evaporated to dryness under a mild nitrogen stream at room temperature. The residue was dissolved in 100 μL of mobile phase, and 10 μL of the solution was supplied to LC−MS/MS for analysis.

LC−MS/MS Analysis. A Shimadzu LC-20A system (Tokyo, Japan), consisting of a CBM-20A system controller, two LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC column oven, and a $DGU\text{-}20A_3$ online degasser, was used for LC−MS/MS analysis. MS/MS detection was performed by an API 3200 triple-stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with electrospray ionization (Turbo Ionspray). Data acquisition and processing were performed with the Analyst 1.4 software (Applied Biosystems).

The HPLC separation was performed on a shim-pack VP-ODS column (2.0 × 150 mm inner diameter, 5 μm) at the column temperature of 40 °C. A mixture of acetonitrile/water (50:50, v/v) was used as the mobile phase, and the flow rate was maintained at 0.2 mL/min.

The mass spectrometer was operated in electrospray negative ionization mode. The ionspray voltage was held at −4.5 kV. The source temperature was set at 550 °C. The pressures of nebulizing gas, curtain gas, and turboheater gas were set at 60, 20, and 60 psi, respectively. Precursor mass and product ion mass for multiple reaction monitoring (MRM) detection, declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and collision cell exit potential (CXP) are listed in Table 1. Figure 2 shows LC−MS/MS chromatograms of 100 ng/mL estrogens monitored at their specific MRM transitions.

Table 1. LC−MS/MS Parameters for the Estrogen Analysis

	MRM traces (m/z)	DP	EP	CEP	CE	CXP
E_1	269.2/145.2	−75	−5	−20	−48	−4
E_2	271.2/145.0	−78	−5	−20	−55	−2
E_3	287.4/145.2	−80	−5	−20	−60	−2
DS	265.2/93.0	−60	−5	−20	−45	−2
HEX	269.2/134.2	−60	−5	−20	−20	−2
DES	279.1/186.0	−60	−5	−20	−30	−2
EE_2	295.2/145.2	−62	−5	−20	−58	−1

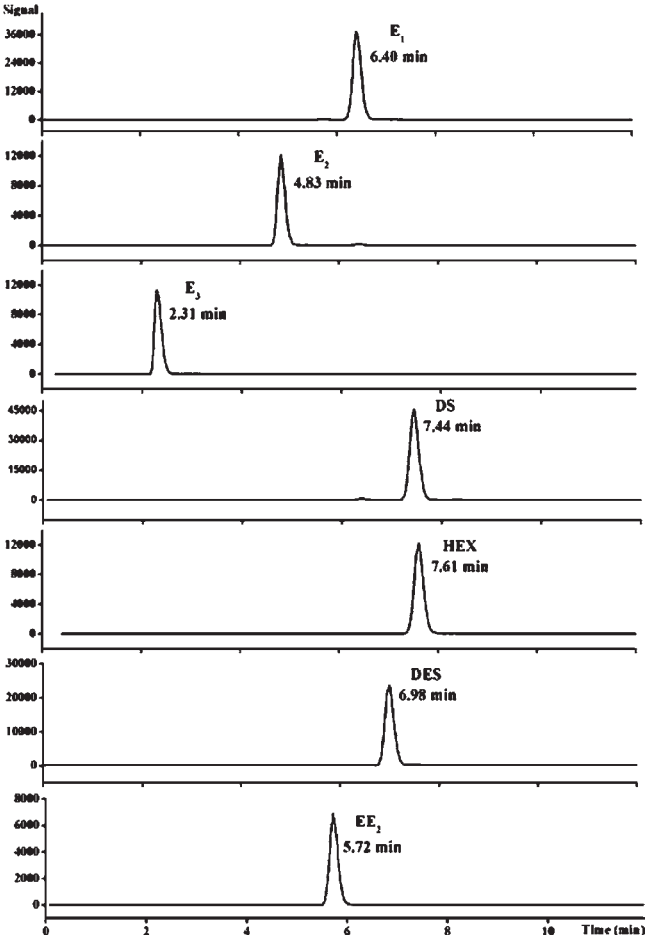

Figure 2. LC−MS/MS chromatograms of seven estrogens obtained from the direct analysis on the standard solution with the concentration of 100 ng/mL for each estrogen.

RESULTS AND DISCUSSION

Characterization of MNPs/PPy. The morphologies and sizes of MNPs and MNPs/PPy were investigated by TEM (see panels a and b of Figure 3). Obviously, MNPs consist exclusively of monodisperse and sphere-like nanoparticles with a mean size of about 65 nm (Figure 3a). In Figure 3b, the dark areas represent MNPs and the light gray phases are the PPy, demonstrating the encapsulation of PPy on the surface of MNPs and the formation of a core−shell architecture. The successful coating of PPy on MNPs should be attributed to the oxidant Fe^{3+} gathering around MNPs and the coordination interactions between MNPs and PPy chains.[29] In addition, it is worth noting that the PPy shell layer is fairly thin (about 10 nm), which should be beneficial to facilitate mass transfer.[18]

At room temperature, typical magnetization curves of MNPs and MNPs/PPy are shown in Figure 3c. Clearly, both magnetic curves have no magnetic hysteresis loops, suggesting they are superparamagnetic.[30] Moreover, MNPs/PPy has a saturation magnetization (M_s) of 27 electromagnetic units (emu)/g, reflecting its good magnetic property.[31] Accordingly, the magnetic MNPs/PPy nanospheres can be separated easily from various media (e.g., milk, water, acetone, etc.) with a permanent magnet. On the other hand, the M_s of MNPs/PPy is obviously lower than that of MNPs (63 emu/g), implying the existence of a significant amount of PPy in MNPs/PPy.[29]

Figure 4 shows the FTIR spectra of MNPs and MNPs/PPy. For MNPs/PPy, the bands at 1455 and 1040 cm^{-1} can be assigned to the C−N ring stretching vibrations of the pyrrole ring. In addition, the peaks of MNPs/PPy at 1166, 886, and 773 cm^{-1} are related to the C−H in-plane and out-plane vibrations. These results are well-consistent with those of other studies,[17,29,32] further verifying the formation of PPy on MNPs.

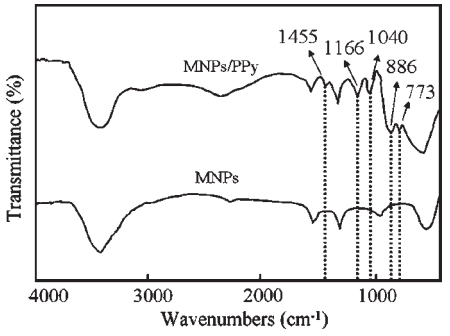

Figure 4. FTIR spectra of MNPs and MNPs/PPy.

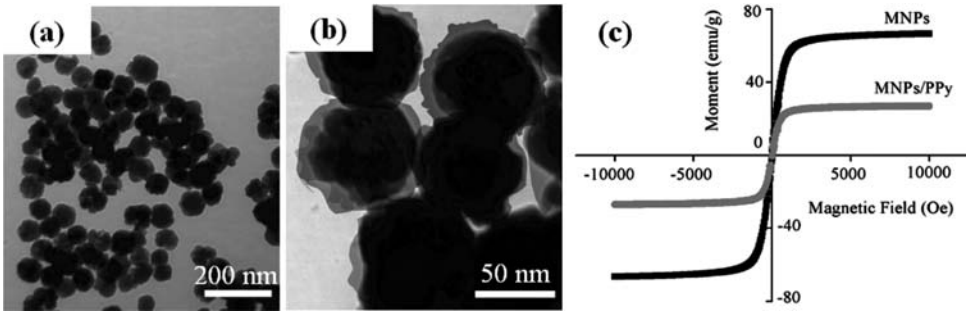

Figure 3. TEM images of (a) MNPs and (b) MNPs/PPy and (c) magnetization curves of two samples.

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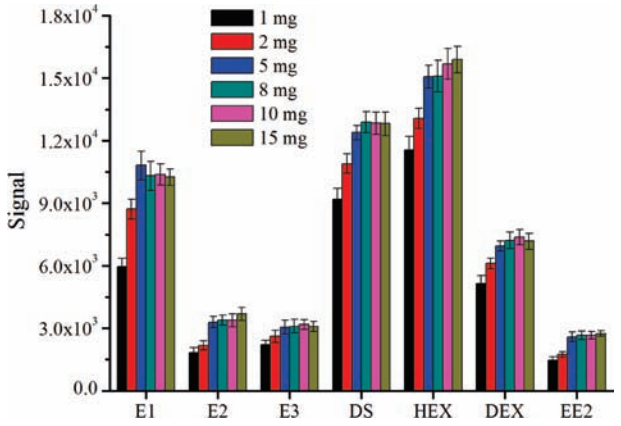

Figure 5. Effect of the MNPs/PPy amount on extraction efficiency with the concentration of 1 ng/mL for each estrogen.

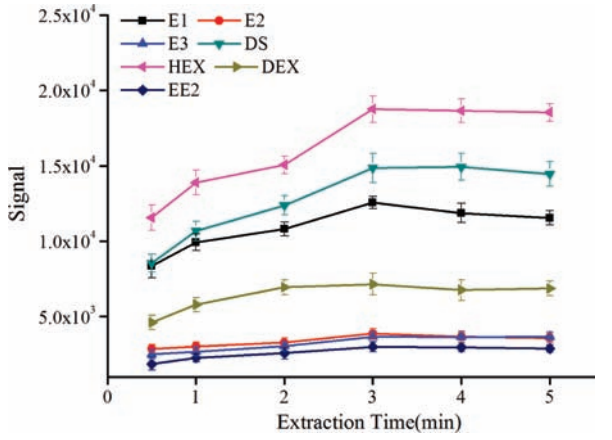

Figure 6. Effect of the extraction time on extraction efficiency with the concentration of 1 ng/mL for each estrogen.

Optimization of Conditions for MSPE. To evaluate the applicability of MNPs/PPy for the separation and enrichment of estrogens in milk samples, the parameters that might affect the performance of the extraction, such as MNPs/PPy amount, pH value, ionic strength, etc., were optimized. The concentration of each estrogen was set at 1 ng/mL. When one parameter was changed, the others were fixed at their optimized values.

MNPs/PPy Amount. To achieve good recovery, different amounts of MNPs/PPy ranging from 1 to 15 mg were applied to extract the estrogens from the milk sample (Figure 5). The results show that the recovery achieved by 5 mg of magnetic sorbent is clearly higher than that achieved by 1 and 2 mg of sorbent but almost the same as those achieved by 8, 10, and 15 mg. Thus, 5 mg was employed in the following experiment.

Extraction Time. The extraction time profiles were conducted by increasing the vortex time from 0.5 to 5 min. It can be seen from Figure 6 that all of the estrogens reach extraction platforms when the time is 3 min. The adsorption reached equilibrium rapidly probably because the PPy coatings on the MNP surface were fairly thin. In this study, the extraction time was set at 3 min.

pH Value and Ionic Strength. The pH optimization was performed in 10 mM phosphate matrix solution over the pH range of 3−8. As shown in Figure 7, no obvious change in

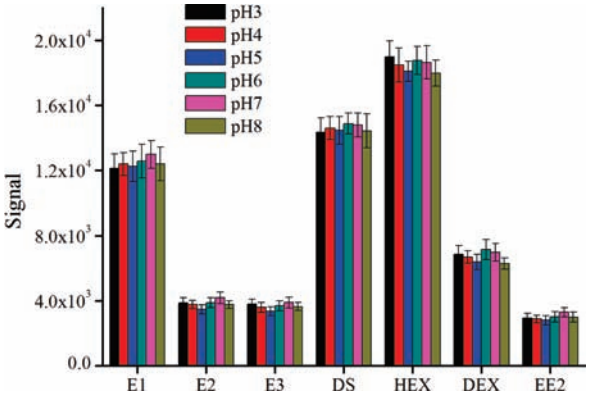

Figure 7. Effect of matrix pH on the elution performance with the concentration of 1 ng/mL for each estrogen.

Table 2. Extraction Response Signal and RSD Value of Estrogens with Three Different Batches of MNPs/PPy Sorbents[a]

	response			
	batch 1	batch 2	batch 3	RSD (%)
E_1	12167	12976	11986	4.3
E_2	3576	4178	3765	8.0
E_3	3316	3890	4234	12.2
DS	15376	14789	13734	5.7
HEX	18765	18056	16987	5.0
DES	7156	6976	6289	6.7
EE_2	2899	3489	2987	10.2

[a] Milli-Q water spiked with estrogens at the concentration of 5 ng/mL.

extraction efficiency is found. This result confirms that the driving force of estrogen extraction should be $\pi-\pi$ and/or hydrophobic interactions between estrogens and MNPs/PPy. In this study, the pH value of the matrix solution was set at 7.

The effect of ionic strength was investigated by gradually increasing the phosphate concentration of the matrix solution from 5 to 30 mM at pH 7. It was found that the extraction efficiencies for all of these estrogens were almost independent of the ionic strength. This result further confirmed that the $\pi-\pi$ and/or hydrophobic interactions played a dominant role to the retention between the estrogens and extraction phase. To accurately control matrix pH, the phosphate concentration was fixed at 10 mM for the following experiments.

Desorption Conditions. The desorption time was optimized by increasing the vortex time from 0.5 to 5 min. It was found that the desorption time had no significant influence on extraction efficiency; therefore, 0.5 min was chosen. We also found that 0.5 min was enough to desorb the extracted estrogens from the magnetic sorbent.

The desorption solvent was optimized to achieve accurate quantification of the analytes. The results showed that 1 mL of acetone can completely elute the extracted extrogens from MNPs/PPy. Because the eluent cannot match LC−MS/MS, it was evaporated to dryness and reconstituted with 100 μL of mobile phase for HPLC, which could increase the sensitivity meanwhile.

Reproducibility of MNPs/PPy. At present, unsatisfactory repeatability is the common limitation of sorbent when different

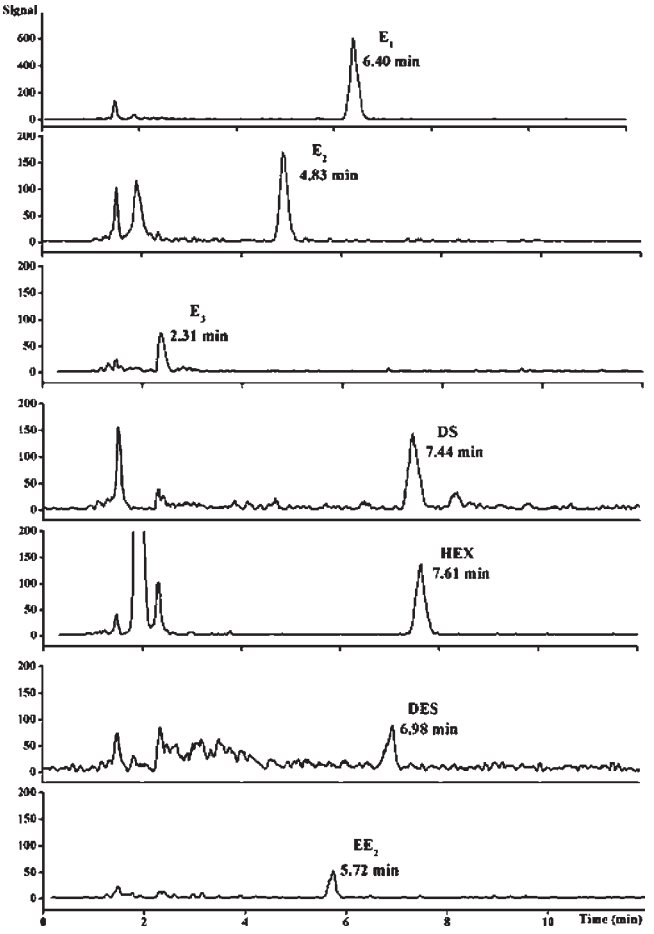

Figure 8. LC−MS/MS chromatograms of seven estrogens extracted by MNPs/PPy from the spiked milk sample containing 0.5 ng/mL of each estrogen.

batches of materials are used. In the present study, the batch-to-batch reproducibility of MNPs/PPy was investigated. Three batches of MNPs/PPy prepared under the same conditions were used for the extraction of estrogens. Table 2 presents the response signal and relative standard deviation (RSD) values of seven estrogens with these three batch sorbents. All of these RSD values are lower than 12.2%, indicating that the laboratory-made sorbent MNPs/PPy has good reproducibility.

Analytical Performance. Estrogens were analyzed under the above-optimized conditions. Figure 8 shows LC−MS/MS chromatograms of the milk sample containing 0.5 ng/mL of each estrogen monitored at its specific MRM transition. It is clearly shown that no interference can be observed even at this low concentration.

The calibration curves were prepared in milk matrix (free of any of the selected estrogens) and constructed by plotting peak area versus concentration with a linear least-squares regression (R^2) analysis. As shown in Table 3, satisfactory correlation coefficients ranging from 0.9974 to 0.9996 are obtained for seven estrogens, which are in the concentration range of 0.08−20 ng/mL (for E_1, E_2, and HEX), 0.2−20 ng/mL (for E_3, EE_2, and DS), and 0.4−40 ng/mL (for DES). The limit of detection (LOD) and limit of quantification (LOQ) were calculated by the concentrations at which signal-to-noise ratios were equal to 3 and 10, respectively. LOD and LOQ data are in the range of

Table 3. Calibration Curve, LOD, and LOQ Data of Seven Estrogens in Milk Samples

	linearity and sensitivity characteristics				
		regression line			
	linear dynamic range (ng/mL)	linear equation	R^2 value	LOD (ng/L)	LOQ (ng/L)
E_1	0.08−20	$y = 886.1 + 30890.6x$	0.9996	5.1	17.1
E_2	0.08−20	$y = 499.7 + 8404.7x$	0.9977	14.9	49.5
E_3	0.2−20	$y = 422.4 + 3894.6x$	0.9984	31.3	104.4
DS	0.2−20	$y = -47.0 + 20175.9x$	0.9996	28.5	94.9
HEX	0.08−20	$y = 404.8 + 5218.0x$	0.9974	15.2	50.5
DES	0.4−40	$y = -110.1 + 8791.5x$	0.9988	66.7	222.2
EE_2	0.08−20	$y = 109.2 + 4400.4x$	0.9977	43.9	146.2

5.1−66.7 and 17.1−222.2 ng/L, respectively (see Table 3). E_1 and DES have the lowest LOD and LOQ values (5.1 and 17.1 ng/L) and the highest values (66.7 and 222.2 ng/L), respectively.

The recoveries were calculated by comparing the extracted amounts of estrogens to those of the samples with the corresponding spiking amounts on calibration curves. The recovery was measured at three different concentrations, and the spiking levels ranged from 0.5 to 20 ng/mL. The recoveries and RSDs are summarized in Table 4; mean recoveries are in the range of 83.4−108.5%.

The intra-assay precision was determined on the same day and consisted of three series and five replicates at each of three concentration levels. Interassay precision was calculated with three replicates at the three fortification levels on three different days. The numerical value used is the RSD of triplicate measurements of the analytes. The results obtained are shown in Table 4. RSDs of intra- and interday ranging from 4.3 to 15.9% and from 5.9 to 17.4% are obtained, respectively.

A comparative study of our developed method to other reported sample preparation procedures was performed, and the results are presented in Table 5. It can be seen that the developed method is very sensitive, and no removal of proteins in milk samples is required prior to extraction in this study.

Applications in Real Samples. To demonstrate the applicability of the method, three kinds of milk samples from a retail market located in Wuhan (China) were analyzed. All samples were analyzed in three replicates. It can be found that sample 1 contains E_3 (0.14 ng/mL), sample 4 contains E_1 (0.07 ng/mL), E_2 (not quantified), E_3 (0.12 ng/mL), and EE_2 (not quantified), and sample 5 contains E_1 (0.05 ng/mL), E_3 (0.23 ng/mL), and HEX (not quantified). In addition, sample 3 contains detectable E_3 and HEX (not quantified), and none of the selected seven estrogens can be found in sample 2. The detailed results are outlined in Table 6.

In summary, nanocomposite MNPs/PPy with a core−shell structure were synthesized and used as the MSPE adsorbent to extract and concentrate estrogens from the milk matrix. It was confirmed that this adsorbent had strong magnetic responsiveness and good extraction capacity toward estrogens in the milk matrix. The extraction could be carried out quickly; the extraction time was clearly shortened to 3 min. Thus, a rapid, simple, and effective method for the analysis of estrogens in milk samples was established by coupling MNPs/PPy-based MSPE with

Table 4. Precisions (Intra- and Interassay) and Recoveries for the Determination of Estrogens in Milk Samples[a]

	intraday precision (RSD %; n = 5)			interday precision (RSD %; n = 3)			recovery (%; n = 3)		
	0.5 ng/mL	5 ng/mL	20 ng/mL	0.5 ng/mL	5 ng/mL	20 ng/mL	0.5 ng/mL	5 ng/mL	20 ng/mL
E_1	8.6	4.4	4.3	10.8	6.4	5.9	95.6 ± 9.7	93.7 ± 7.1	101.7 ± 4.2
E_2	9.2	10.5	7.6	13.9	12.3	8.0	105.1 ± 9.2	92.4 ± 10.3	95.3 ± 6.8
E_3	8.3	9.4	5.9	12.7	10.5	8.3	89.8 ± 14.9	83.4 ± 9.2	105.1 ± 7.3
DS	15.9	12.3	8.4	17.4	11.9	10.3	107.7 ± 8.6	97.7 ± 11.6	93.8 ± 10.0
HEX	13.2	12.1	9.3	12.9	13.5	7.8	99.6 ± 9.8	88.1 ± 9.5	93.3 ± 4.3
DES	13.9	7.1	6.9	16.3	9.8	7.2	93.5 ± 15.4	108.5 ± 8.2	90.7 ± 7.5
EE_2	14.9	10.6	9.7	13.2	12.8	10.2	92.1 ± 13.1	84.3 ± 9.7	91.7 ± 4.5

[a] Recoveries from three experiments are given as average values ± RSDs.

Table 5. Comparison of the Sample Preparation Procedures and LODs between Different Methods for Their Application in Milk Samples

estrogens	matrix	extraction	protein removal	determination	LODs (ng/L)[a]	reference
E_1, E_2, E_3, EE_2, E_1-3S, E_2-3S, E_2-17S, EE_2-3G, E_1-3G, E_2-3G, E_3-3S, E_2-3S-17S, E_2-3G-17S, E_2-3G-17G	milk	Oasis HLB SPE	protein precipitation	LC−MS/MS	2−70	8
E_1, E_2, E_3, EE_2, DES, BPA	milk	triacontyl-bonded silica as online SPE	protein precipitation	LC−MS	80−300	7
estriol, 17β-estradiol, estrone, dienestrol, diethylstilbestrol, hexestrol, trenbolone, nandrolone, testosterone, methyltestosterone, stanzolol	milk	HLB SPE	protein precipitation	LC−ESI−MS/MS	2−100	9
E_1, E_2, E_3, DS, HEX, DES, EE_2	milk	MNPs/PPy-based MSPE	no removal of protein	LC−MS/MS	5.1−66.7	this work

[a] The unit in ref 9 was ng/kg.

Table 6. Determination of Estrogens in Five Milk Samples[a]

	sample 1	sample 2	sample 3	sample 4	sample 5
	detected concentration (ng/mL, RSD %; n = 3)	detected concentration (ng/mL, RSD %; n = 3)	detected concentration (ng/mL, RSD %; n = 3)	detected concentration (ng/mL, RSD %; n = 3)	detected concentration (ng/mL, RSD %; n = 3)
E_1	nd	nd	nd	0.07 (12.6)	0.05 (9.5)
E_2	nd	nd	nd	nq	nd
E_3	0.14 (16.3)	nd	nq	0.12 (10.7)	0.23 (8.9)
DS	nd	nd	nd	nd	nd
HEX	nd	nd	nq	nd	nq
DES	nd	nd	nd	nd	nd
EE_2	nd	nd	nd	nq	nd

[a] nd, not detected; nq, not quantified; sample 2 is also the sample that was used as a blank milk for calibration and validation.

LC−MS/MS. The LOQs and LODs of estrogens were in the range of 5.1−66.7 and 17.1−222.2 ng/L, respectively, with inter- and intraday precisions less than 17.4% at three different concentrations and recoveries between 83.4 and 108.5%.

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