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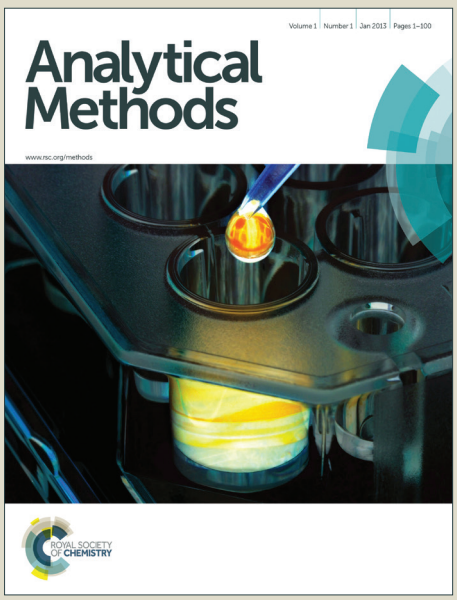

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Chitosan– polypyrrole @ Fe_3O_4 nanocomposite for magnetic solid-phase extraction of macrolides from swine urine samples

Qie Gen Liao, Li Fang Hu and Lin Guang Luo*

Laboratory of quality & safety risk assessment of livestock and poultry products (Nanchang), Ministry of agriculture, Agricultural product quality safety and standards institute, Jiangxi academy of agricultural sciences, Nanchang, 330200, P R China. E-mail: luolinguang2014@163.com.

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Abstract In this paper, a new method was established for the determination of four macrolides (azithromycin, roxithromycin, clarithromycin and tylosin tartrate) from swine urine samples by magnetic solid-phase extraction coupled with liquid chromatography tandem mass spectrometry. The extraction adsorbent was synthesized to form a chitosan-polypyrrole (CS-PPy) @ Fe_3O_4 core –shell magnetic nanocomposite. Main factors influencing the extraction efficiency including amount of adsorbent, solution pH, extraction time, and volume of desorption solution were studied and optimized. Under the optimal conditions, recoveries of the spiked samples ranged from 76 to 84 % with the relative standard deviations lower than 10 %. The limits of detection were 0.04 and 0.2 μg L^{-1} for azithromycin, roxithromycin, clarithromycinand tylosin, respectively. The proposed method was successfully applied for selective and efficient determination of macrolides from swine urine samples.

Key words: Liquid chromatography tandem mass spectrometry; Magnetic solid-phase extraction; Macrolides; Swine urine; Chitosan- polypyrrole (CS-PPy) @ Fe_3O_4 core –shell magnetic nanocomposite

1 Introduction

Macrolide antibiotics are active agents against Gram-positive and some Gram-negative bacteria, and are widely used in human and veterinary for both therapeutic and prophylactic treatments against bacterial infections such as mastitis. [1-3] Macrolides are also employed as growth promoters in stock farming at subclinical doses in food producing animals.[4] The incorrect use of these drugs can leave residues in food products and this can have such undesirable effects on consumer health as the development of allergic reactions, the appearance of resistant bacteria and even cross-resistance to other antibiotics with similar structures or mechanisms of action.[5]

Different methods have been proposed for analyzing macrolides.[6-11] Among these methods, LC-MS/MS is the most popular technique because of its sensitivity, specificity, and its ability to identify unknowns. The difficulties in establishing analytical methods for the analysis of macrolides in food and biological samples are mainly attributable to the complexity of the sample matrices and the low concentrations of the macrolides in the samples. After oral administration of macrolides one main elimination route is urinary excretion.[12] Pharmacokinetic studies suggested that macrolide antibiotics are not excessively metabolised; the respective parent compounds are therefore predominant in excreta.[13] In fact many control systems in slaughterhouses are based on analysis of urine samples, because this matrix also has the advantage of being one of the few matrices available while the animals are still alive. However, drug residue concentrations in the urine are often low, a preconcentration step is generally required for the determination of macrolides in complex sample matrices.

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 .[14-18] 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 can 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.[15] However, to the best of our knowledge, until now MSPE has not been applied to macrolide extraction from swine urine samples. On the other hand, core–shell magnetic composites have attracted considerable attention can provide favorable biocompatibility and enough functional groups for adsorption, and protect magnetic nanoparticles from leaching in an acidic environment. [19-21]Polypyrrole (PPy) as a modified shell, has been studied extensively in its great potential application in many fields.[21-25]

In this study, chitosan-polypyrrole (CS-PPy) @ Fe_3O_4 core–shell magnetic nanocomposite was synthesized for the MSPE of macrolides from swine urine samples. The analyte concentration in the eluent was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) detection. Several factors related to MSPE efficiencies, such as type and amount of sorbent, extraction time, sample pH, and desorption conditions were investigated. The developed method was applied to the analysis of macrolide from swine urine samples.

2 Experimental

2.1 Chemicals and standard solutions

Chitosan (CS), ammonium peroxydisulfate (APS), hydrochloric acid, sodium hydroxide, acetic acid, formic acid, ethyl acetate, $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$ and pyrrole were analytical grades and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). An N35-gradeNdFeB magnet (60×20×10 mm) was used for magnetic separation, which was purchased from Guanneng Magnetic (Yinzhou, Ningbo, China). Azithromycin (AZI), roxithromycin(ROX), clarithromycin(CLA) and tylosin tartrate(TYL) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). A

standard stock solution was prepared by dissolving 10 mg of each standard in10 mL of acetonitrile and stored in dark at 4◦C. Working solutions were obtained daily by appropriately diluting the stock solutions with acetonitrile. Ultrapure water was obtained from a Milli-Q system from Millipore (Milford, MA, USA).

2.2 Swine urine samples

All urine samples were collected from different breeding base in Jiangxi (China) and stored at 20 °C. One urine sample was checked to be free of any of the selected macrolides and used as blank urine for calibration and validation purposes. The four macrolides were directly spiked into 5 mL of urine sample over a range of 2.0-10 ng mL^{-1}. After mixing evenly, the sample was diluted to 10 mL with ammonium acetate buffer (0.1 mol L^{-1}, pH 10.0) before use. Blank urine samples were prepared in the same way as described above but without the analyte-spiking step.

2.3 Synthesis of Fe_3O_4, CS @ Fe_3O_4 and CS–PPy @ Fe_3O_4 magnetic nanocomposite

Briefly, 5.2 g of $FeCl_3 \cdot 6H_2O$ and 2 g $FeCl_2 \cdot 4H_2O$ and 10.0 mL concentrated HCl were dissolved in 160 mL water under theN_2 gas. The mixture was stirred vigorously while the temperature was increased to 60°C. A stream of air was bubbled in the mixture whilst a NaOH solution (10%) was added to adjust pH value to 10. After 1.0 h, the magnetic precipitates were isolated from the solvent by a permanent magnet and washed several times with degassed water.

First, 4.16 g $FeCl_3 \cdot 6H_2O$ and 1.6 g $FeCl_2 \cdot 4H_2O$ were dissolved into 200 mL acetic acid aqueous solution (0.25 % v/v) containing 2.5 g L^{-1} CS. After being stirred for 1 h at 40 °C under the nitrogen atmosphere, then sodium hydroxide solution (10%) was added drop by drop into the solution under vigorous stirring for 1 h. On the surface of Fe_3O_4 nanoparticles the mixed hemimicelle of CS formed. Finally, the resulted brown precipitates were collected using the permanent magnet and washed consecutively with methanol and doubly distilled water.

The synthesis procedure for CS– PPy magnetic nanocomposite was performed

according to self-assembly approach .[21] The CS @ Fe_3O_4 has hydrophobicand hydrophilic moieties so they could facilitate the dissolution of pyrrole. The CS–PPy magnetic nanocomposites were synthesized by addition of above-mentioned CS @ Fe_3O_4 to 160 mL water containing 5 mL pyrrole stirring for 1 h at room temperature under the nitrogen atmosphere. Then suitable amount of APS, as initiator, was added to the solution and stirred for 4 h at room temperature and CS–PPy magnetic nanocomposites were obtained. The black CS–PPy magnetic nanocomposite was collected using the permanent magnet and washed three times by double distilled water and methanol. The washing procedure was continued until the filtrate became colorless.

The size and morphology of the magnetic nanocomposite were investigated by using a FEI Quanta 200 scanning electron microscope (SEM) (Philips-FEI, Netherlands). The magnetic properties were analyzed by using a vibrating sample magnetometer (Lake Shore 7410, USA). The Fourier transform infrared spectroscopy (FTIR) spectra (400–4000 cm^{-1}) were recorded using KBr pellets by Agilent 5700 FTIR spectrophotometer (Agilent technologies, USA). The thermal degradation/stability of the nanocomposite was studied with a thermo-gravimetric analysis; PE Dimand TG/DTA (PerkinElemer MA, USA). Analysis was performed from the room temperature to 740 ◦C at a heating rate of 10 ◦C min^{-1} in an air atmosphere.

2.4 MSPE procedure

In the proposed extraction procedure (Fig.1), fifteen milligrams magnetic nanocomposite and 1.5 g NaCl were dispersed into 10.0 mL of swine urine sample under shaking for 3 min. Then, the NdFeB magnet was held at the bottom of the flask and the adsorbent was isolated from the suspension. After about 5 s, the suspension became clear and was decanted. The residual sorbent was eluted with 5.0 mL of acetonitrile/methanol (1:1, v/v) to desorb the adsorbed analytes. Subsequently, desorption solution was dried under a mild stream of nitrogen at 40 °C. Finally, the residue was reconstituted in 1.0 mL of acetonitrile/water (1:9, v/v), and 10.0 µL was used for LC-MS/MS analysis. After

desorbed the adsorbed analytes from the magnetic sorbent, the sorbent was recycled by washing with 5.0 mL acetonitrile/methanol(1:1, v/v) twice.

2.5 LC-MS/MS Analysis

The LC-MS/MS analysis was achieved using an Agilent 1290 HPLC series and an Agilent 6460A triple-quadrupole mass spectrometer equipped with an electrospray (ESI) ionization interface (Agilent technologies, USA). For instrument control, masshunter workstation software data acquisition for triple quad B.04.01 (B4114.SP5) and qualitative analysis version B.05.00/build 5.0.519.13 were used for data acquisition and processing. Sample injection volume was 10 μL. A reversed phase Eclipse XDB C18 column (1.8 μm particle size, 2.1 mm×100 mm) from Agilent technologies was employed for HPLC separation at 40 °C. The multi-class nature of the MCs showed preferably positive ionization and were detected as $[M + H]^+$. For compounds detected in ESI+ mode, a binary mobile phase at the flow rate of 0.3mL min^{-1} was composed of water containing 0.1% formic acid (v/v) (A) and acetonitrile. The system was programmed to deliver the following linear gradient: 0min (80% A, 20% B), 4.0 min (40% A, 60% B), 5.0 min (0% A,100% B), 5.1 min (80% A, 20% B), 6.0 min (80% A, 20% B).

The MS determination was performed in ESI^+ mode (using the optimized MS instrument parameters obtained by the tuning) combined with monitoring of the two most abundant MS/MS (precursor-product) ion transitions. Table S1 in Supporting Information gave analyte-specific MS/MS conditions and LC retention times for the LC-amenable analytes. The MS source conditions were as follows: source temperature of 100 °C, desolvation gas temperature of 350 °C, desolvation gas of 11.0 L min^{-1}, nebulizer gas (N_2) pressure of 40.0 psi.

3 Results and discussion

3.1 Characterization of the prepared nanocomposite

The SEM images of the Fe_3O_4 (Fig. 2a), CS@ Fe_3O_4 (Fig. 2b) and the CS–PPy @

Fe_3O_4 magnetic nanocomposite (Fig.2c) show a more porous structure for the latter composite.

The magnetization curves show that CS@ Fe_3O_4 and CS–PPy @ Fe_3O_4 exhibit typical superparamagnetic behavior due to no hysteresis (Fig. 2d). There is no remanence and coercivity, suggesting that such NPs are superparamagnetic. The saturation intensities of magnetization are 59.3 emu g^{-1} for CS @ Fe_3O_4 and 41.6 emu g^{-1} for CS–PPy @ Fe_3O_4, which are sufficient for magnetic separation with a conventional magnet. Apparently, the nonmagnetic PPy on the CS@ Fe_3O_4 result in the decrease of the magnetic strength for CS–PPy @ Fe_3O_4. As a result, the CS–PPy @ Fe_3O_4 nanocomposite in their homogeneous dispersion show fast movement to the applied magnetic field and redisperse quickly with a slight shake once the magnetic field is removed (insetin Fig. 2d). It suggests that the nanocomposite possess excellent magnetic responsivity and redispersibility, which is an advantage totheir applications.

The FTIR spectra of Fe_3O_4, CS @ Fe_3O_4 and CS–PPy @ Fe_3O_4 nanocomposite are shown in Figure S1 of Supporting Information. All FTIR spectra have a peak at 580 cm^{-1} that corresponds to Fe–O stretching band. The characteristicabsorption bands for CS at 3422 cm^{-1} (O–H and N–H stretching vibrations), 2866 cm^{-1}(C–H stretching vibrations), 1634 cm−1(N–H bending vibrations), and 1072 cm^{-1}(C–O–C stretching vibrations). The characteristics of pristine PPy and the peak at 3420 cm^{-1} is attributed to N–H band whilethe peaks at 1528 and 1486 cm^{-1} are attributed to C=N and C=C stretching mode for the quinoid and benzenoid rings. The peaks at 580 cm^{-1}, 1072 cm^{-1}, 2866 cm^{-1}, 1528 cm^{-1} and 1486 cm^{-1} showed that CS and PPy had held on the surface of Fe_3O_4 nanoparticles.

The indication of the coating formation on the Fe_3O_4 surface and their thermal stability can be obtained from TGA/DTG analysis, as shown in Fig. 3S. It can be observed from Fig. 3S that CS and CS-PPy coating of the Fe_3O_4 nanoparticles started to decompose at the temperature of 200 ◦C and undergoes different decomposition patterns.

In the first step of decomposition process, a nonlinear continuous weight loss in the temperature range of 200–600 ◦C was observed on the surface of CS @ Fe_3O_4 and CS–PPy @ Fe_3O_4 nanocomposite. A rapid weight loss from 600 to 700 ◦C occurred on the surface of CS–PPy @ Fe_3O_4. Upon heating in TGA, CS @ Fe_3O_4 and CS–PPy @ Fe_3O_4 nanocomposite have a weight loss of about 29% and 57%, showing that the CS and PPy have self-assembled on the surface of Fe_3O_4. In addition, a mutation point at 673 ◦C in the DTG curve of CS–PPy @ Fe_3O_4 nanocomposite shows also that PPy has held on the surface of Fe_3O_4 nanoparticles.

3.2 Optimization of extraction conditions

In order to achieve satisfactory extraction efficiency of the proposed MSPE procedure for the macrolides, several parameters that may affect the extraction efficiency were optimized, such as, the amount of the sorbent, desorption solvent, solution volume and the extraction time. The influences of all these parameters were evaluated in terms of recovery rate. The optimization experiments were conducted using spiked standard macrolides solution containing 2.0 μg L^{-1} of each analyte. Each experiment was performed in triplicate.

3.2.1 Effect of the type of sorbent

The morphology and structure of sorbent are key factors in the extraction strategy. In this study, the extraction capabilities of magnetic nanocomposite coated with CS and CS–PPy were examined by extracting macrolides, as model compounds, from aquatic media. According to the obtained results from Fig. S2, the recoveries of macrolides had a significant increase in the presence of pyrrole, which indicated that PPy had a vital role in the extraction process.

3.2.2 Effect of solution pH

The pH of sample solution could influence the extraction performance of the analytes by changing both the existing forms of the target compounds and the species and

density of charges on the adsorbent surface. The pH values of the sample solutions were adjusted with different pH ammonium acetate buffers. As shown in Fig. 3A, the extraction recoveries of the macrolides is acceptable in the whole pH range of 6.0–11.0, demonstrating the highest adsorption rates were generally observed at pH 10. Thus, pH 10 was considered the optimum pH.

3.2.3 Effect of the sorbent amount and extraction time

To appraise the effect of sorbent quantity on the extraction efficiency, different amounts of sorbent within the range of 2.0–20 mg were added to the solution. The result, as illustrated in Fig. 3B, shows that the best extraction efficiency of macrolides could be obtained using 15 mg of the sorbent. Compared to the ordinary sorbents, nano-sized sorbents have higher surface areas, therefore satisfactory results can be obtained by lower amounts of nano-sized sorbents. Also, due to the shorter diffusion route for the sorbent and the magnetically assisted separation of the sorbent from the sample solutions, the extraction of target analytes can be achieved in a shorter time.

To reveal the effect of extraction time on the extraction efficiency of the drugs, the extraction times were varied in the range of 0.5-5 min. It was found that extending the extraction time more than 3 min had no effect on peak area, so 3 min was selected as extraction time. Such a fast adsorption rate could be attributed to the absence of an internal diffusion resistance, since the adsorption of the macrolides occurred only on the surface of the sorbent.

3.2.4 Effect of ionic strength

Generally, the solubility of the hydrophobic compounds decreases with increasing ionic strength in aqueous solution. This “saltingout” effect may slightly enhance their hydrophobic interactions with sorbent. On the other hand, the aggregation of sorbent could be enhanced by the increase of ionic strength, namely “squeezing-out” effect, since the repulsive force between the sorbent would become smaller due to the penetration of

the counter-ions into the diffuse double layer surrounding the sorbent particles. To examine the impacts of ionic strength, experiments were performed by addition of NaCl salt in water samples from 0 to 20 % (w/v) prior to extraction. An increase of ionic strength had a positive effect on the adsorption of estrogens by the sorbent (Fig. 4A), suggesting that within the ionic strength range studied, the contribution of the salting-out effect to macrolides was higher than that of the squeezing-out effect to sorbent. Thus, the addition of 15 % sodium chloride was expected to exert a positive effect on the adsorption of macrolides by sorbent.

3.2.5 Desorption conditions

The desorption solvent is crucial for obtaining a satisfactory desorption efficiency for the analytes. Several organic solvents including ethyl acetate, methanol, acetonitrile and methanol/acetonitrile(1:1, v/v) were used to elute the macrolides from the magnetic sorbent. As shown in Fig. 4B, acetonitrile gained the highest desorption efficiency for AZI, however, methanol gained the highest desorption efficiency for other macrolides. Thus, the methanol/acetonitrile(1:1, v/v) was selected as the desorption solvent. Furthermore, the influence of the elution volume of acetonitrile from 2 to 10 mL on desorption efficiency was also studied. According to the experiments, all the analytes could be completely desorbed from the sorbent by rinsing with 5 mL of acetonitrile. Desorption times were evaluated within the range of 1–5 min. The results showed that the time of 2 min appeared to be the optimum value for the elution of analytes.

3.2.6 Reusability of the sorbents

In order to investigate the recycling of the magnetic sorbents, the sorbent was rinsed with 5 mL of acetonitrile/methanol (1:1, v/v) twice before application in the next time. After 10 times of recycling, there was no obvious decrease or increase for the recoveries of analytes. The results indicated that the sorbent was reusable with no analyte carryover during MSPE procedure, showing good reusability.

3.3 Method evaluation

The linear range of the method was established using blank urine samples spiked with the target compounds at six levels from 0.5 to 20.0 μg L^{-1} for the macrolides, each injected in triplicate. The square of correlation coefficient (R^2) was between 0.9975 and 0.9982. Limits of detection (LOD) and of quantification (LOQ) were calculated by extrapolation of the concentrations giving a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LODs ranged from 0.04 to 0.2 μg L^{-1} while the LOQs ranged from 0.1 to 0.5 μg L^{-1} (Table 1). For recovery studies, blank water samples were spiked with PCBs at three concentration levels of 1.0, 2.0 and 5.0 μg L^{-1}, and the intra-day recoveries obtained ranged from 78 % to 83 % at all spiked levels, while the intre-day recoveries obtained ranged from 76 % to 81 % at all spiked levels. The intra-day repeatability of the method expressed as relative standard deviations (RSDs) for six replicates ranged from 3% to 6%, while the intre-day repeatability of the method expressed as RSDs for six replicates ranged from 4% to 8%.

3.4 Determination of macrolides in swine urine samples

In order to evaluate the applicability of the proposed method, a survey on macrolides swine urine samples collected in breeding base was performed. The results indicated that AZI were found in three swine urine samples with their concentrations ranging from 2.7 μg L^{-1} to 4.6 μg L^{-1}, and other samples were not contaminated by macrolides. According to Table 2, the proposed method considerably accelerated the sample preparation procedure and chromatographic separation time because only 20 min was required to the sample preparation and 6.0 min was required to separate macrolides with high resolution. Moreover, the magnetic adsorbent could be easily and quickly isolated within 5s and recycled from urine samples with an external magnetic field. Additionally, the LODs of the proposed method were better than those obtained with other methods.

4 Conclusion

In the present work, CS-PPy @ Fe_3O_4 core–shell magnetic nanocomposite was used

as the sorbent for the MSPE of macrolides at trace levels in swine urine samples. Combined with LC–MS/MS, the developed method offered excellent sensitivity, wide linear range, and ease of operational, as well as satisfactory recovery and repeatability under optimized conditions. The method was successfully used to analyze real swine urine samples.

Acknowledgments

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Figure and Table captions

Fig. 1 Procedure of magnetic solid-phase extraction of macrolides in swine urine samples.

Fig. 2 (a) SEM images of Fe_3O_4; (b) CS@Fe_3O_4; (c) CS-PPy@Fe_3O_4; (d) magnetic curves of CS@Fe_3O_4 and CS-PPy@Fe_3O_4. The inset shows the separation–redispersion process of CS-PPy@Fe_3O_4.

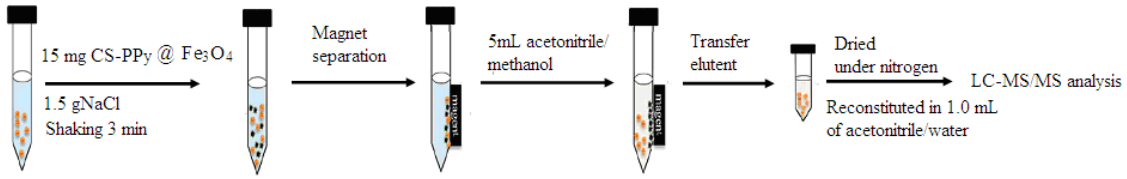
Fig. 3 Optimization of the MSPE procedure. (A) Effect of sample solution pH on the recoveries of macrolides. (B) Effect of the amount of the sorbent on the recoveries of macrolides.

Fig. 4 Optimization of the MSPE procedure. (A) Effect of salt concentration on the recoveries of macrolides. (B) Effect of desorption solvents on the recoveries of macrolides.

Table 1

Analytical performance in swine urine samples

Table 2 Comparison of the proposed MSPE method with previous methods for the determination of the macrolides.



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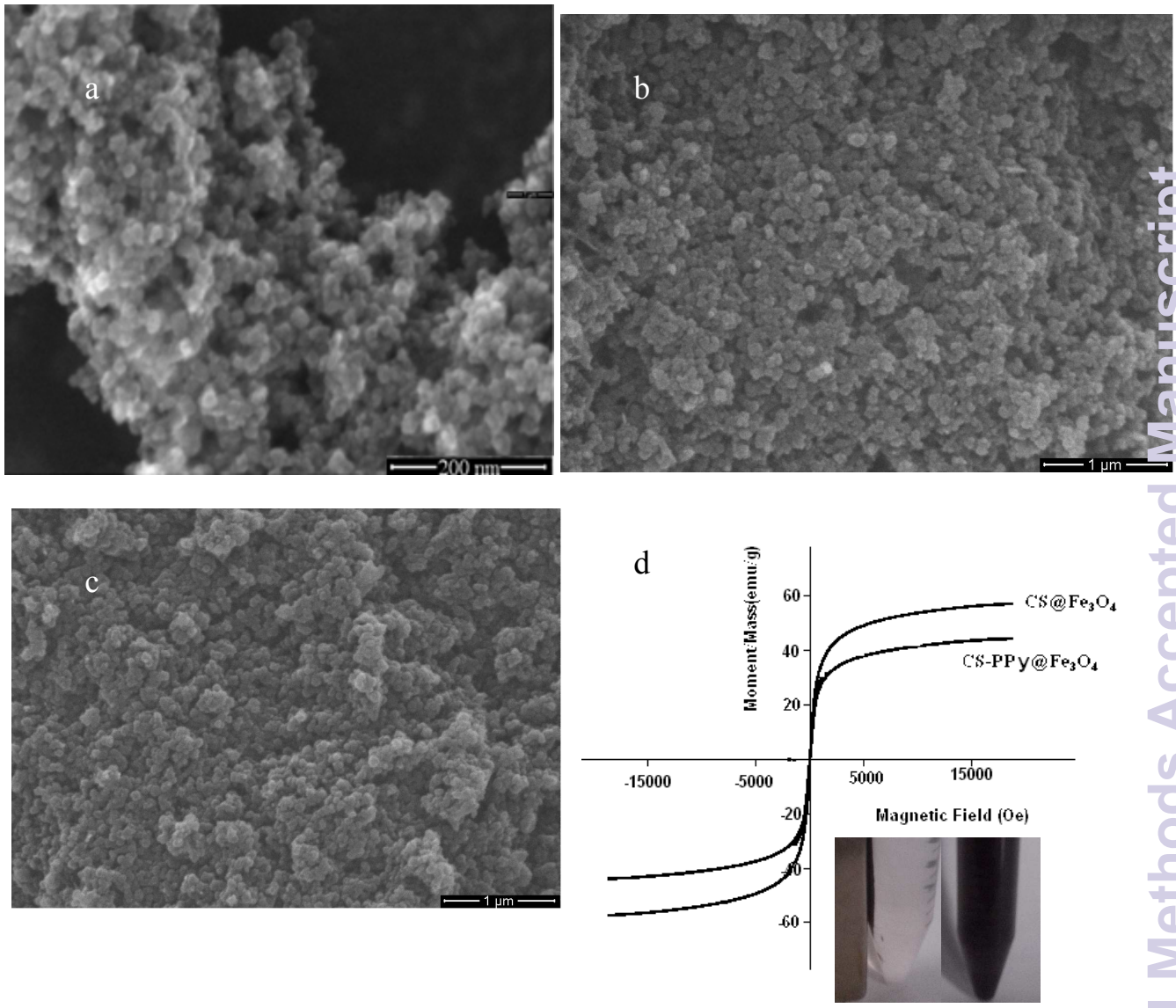

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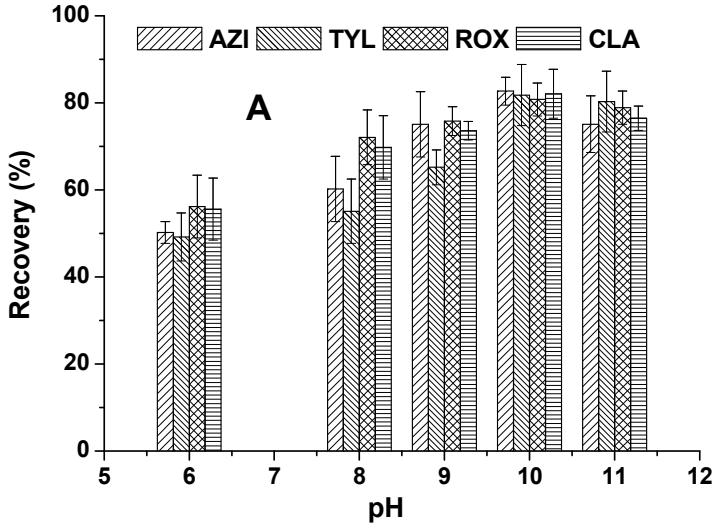

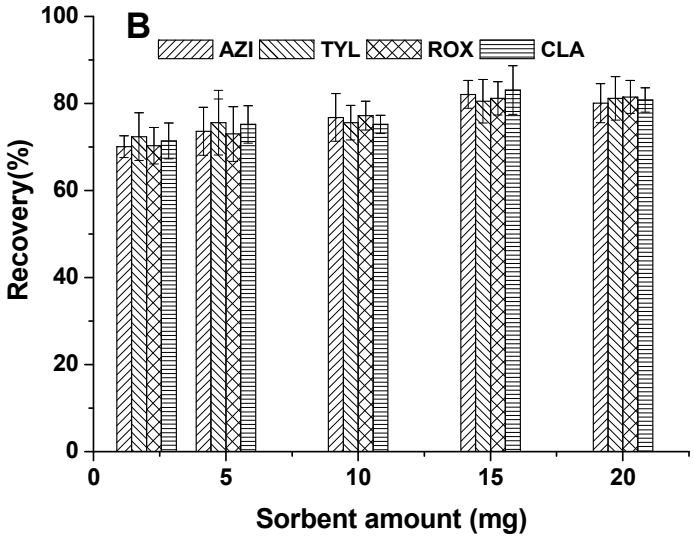

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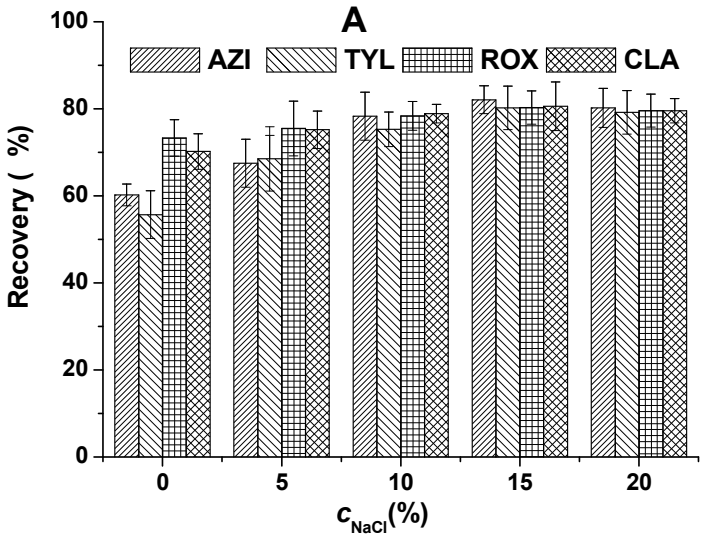

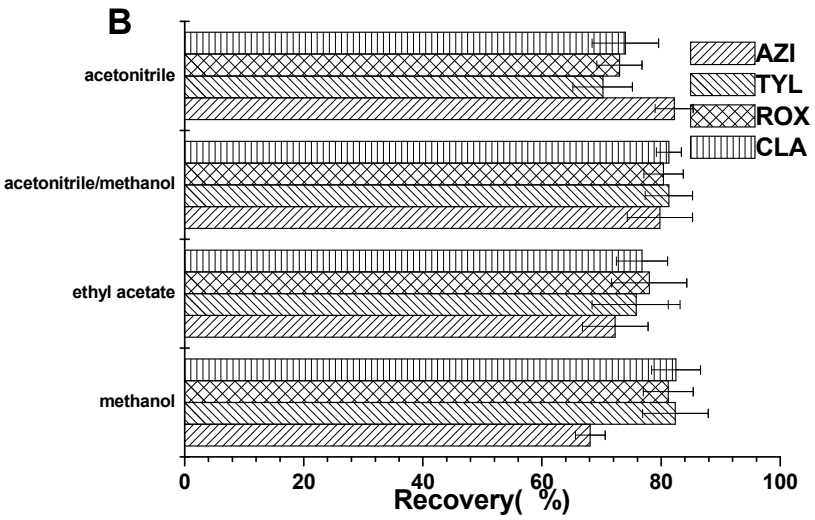

Fig. 3 Optimization of the MSPE procedure. (A) Effect of salt concentration on the recoveries of macrolides. (B) Effect of desorption solvents on the recoveries of macrolides.

Table 1

Analytical performance in swine urine samples.

Analyte	LOD, μg L^{-1}	LOQ, μg L^{-1}	Intra-day recovery ± RSD (%) (n = 6)			Inter-day recovery ± RSD (%) (n = 6)		
			1.0 μg L^{-1}	2.0 μg L^{-1}	5.0 μg L^{-1}	1.0 μg L^{-1}	2.0 μg L^{-1}	5.0 μg L^{-1}
AZI	0.2	0.5	79±5	81±6	83±4	77±7	78±6	81±5
ROX	0.04	0.1	78±6	81±4	82±5	76±8	79±5	80±7
CLA	0.2	0.5	81±4	83±5	82±3	79±5	81±8	80±6
TYL	0.04	0.1	81±4	82±5	81±3	79±5	80±8	81±6

Table 2 Comparison of the proposed MSPE method with previous methods for the determination of the macrolides.

Mehtods	matrix	LODs/LOQs(ng L^{-1}/ ng kg^{-1})	Recovery (%)	Sample preparation time (min)	Chromatographic separation time (min)	Reference
MSPD[a]	sheep milk	24.1	74-97	>45	32	[6]
DLLME-SFO[b]	human urine	10-40	100	>20	20	[7]
DLLE[c]	porcine and bovine urine	70 or 100	69.7-96.	60	30	[11]
MSPE	swine urine	0.04 or 0.2	76-84	<20	6	Proposed method

[a]MSPD, matrix solid phase dispersion; [b]DLLME-SFO, dispersive liquid–liquid microextraction based on the solidification of floating organic droplets; [c]DLLE, double liquid–liquid extraction

1. A more porous structure chitosan- polypyrrole (CS-PPy)@Fe_3O_4 nanocomposite was controllably synthesized.

2. The CS-PPy@Fe_3O_4 nanocomposite showed high extraction efficiencies toward macrolides

3. An effective MSPE procedure with CS-PPy@Fe_3O_4 nanocomposite has been developed for extraction of four macrolides.

4. An effective MSPE-LC–MS/MS method for determination of macrolides in swine urine samples has been developed.