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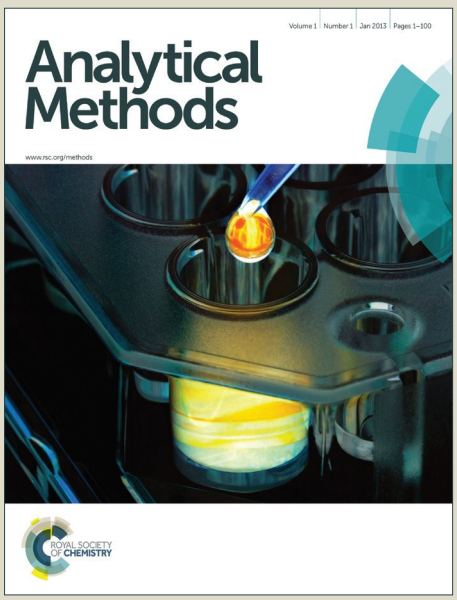

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Fast clean-up and selective enrichment of florfenicol in milk by restricted access media - molecularly imprinted magnetic microspheres based on surface-initiated photoiniferter – mediated polymerization

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A novel restricted access media-molecularly imprinted magnetic microsphere (RAM-MIMM) was prepared by initiator-transfer agent-terminator (iniferter) method. The iniferter was synthesized through the magnetic nanoparticles were coated with silica-gel, modified with amino-group and reaction with 4-(chloromethyl)benzoyl chloride and sodium diethyldithiocarbamate. The molecularly imprinted magnetic microsphere (MIMM) was prepared using florfenicol (FF) as the template, itaconic acid (ITA) as the functional monomer, ethylene glycol dimethacrylate as the cross-linker, iniferter as surface initiator in anhydrous ethanol solvent. The hydrophilic external layer of the MIMM were formed by iniferter polymerization using glycidyl methacrylate (GMA) as monomer, and then the hydrolysis of the linear poly(GMA).The obtained RAM-MIMMs were characterized by TEM, TGA, FT-IR and adsorption experiments. The Fe_3O_4 magnetic microspheres were demonstrated with the average diameters around 230 nm and the coating thickness in the range of 20-30 nm. The RAM-MIMMs exhibited high selectivity (1.68) of the imprinted cavities and hydrophilicity of the external surface with water compatibility and exclusion biomacromolecules. The RAM-MIMMs were used for magnetic dispersion extraction of FF from milk samples. The average recoveries were obtained in the range of 85.4-94.7% with precision 2.95-4.97%. The limits of detection and quantitation of the proposed method were in the range of 4.40-18.31 μg kg^{-1} and 14.66-61.02 μg kg^{-1}, respectively. The proposed method was successfully applied to fast clean-up and selective enrichment of florfenicol in milk.

Introduction

Florfenicol (FF), is a structural analogue of chloramphenicol (CAP), is a synthetic broad-spectrum antibacterial agent in veterinary treatment of bacterial diseases.[1] What's more, owing to the ban of the use of CAP in food-producing animals, FF is used increasingly in aquaculture, livestock, and poultry to treat diseases, and hence is potential antibacterial drugs for food-producing animals. Compared with the same kind analogues of CAP and thiamphenicol (TAP), FF has the advantage of good antibacterial activity and no potential of regenerative anemia. Although FF is a more secure drug than CAP and TAP, its use in animal husbandry has the potential to result in the presence of residues in tissues and the increased emergence of resistance of pathogenic bacteria that could have potential health risks to humans.[2] Given this situation, the maximum residue limits in various tissues are fixed by many countries or organizations, and China's Ministry of Agriculture has defined a maximum residue limit (MRL) for FF in food of animal origin at a level of 0.1 mg kg^{-1} to date.[3]

Therefore, the determination of FF has already become the worldwide hot subject. Many different methods have been described for the determination of FF in animal tissues including HPLC,[4] LC-MS/MS,[5] GC[6] and GC-MS.[7] Although these methods can produce satisfactory results for detecting FF, sample pretreatment is necessary for the determination of FF. So far, solid-phase extraction (SPE) is the most widely used sample pretreatment techniques.[8] Adsorbent is a key factor in solid-phase extraction, and molecular imprinting technique is an effective method for the preparation of selective adsorbent. The florfenicol-imprinted polymers have been developed for selective extraction of veterinary drug residue from complex food samples.[8-10] However, some problems still exist in the MIP-SPE, such as tedious column packing procedure, low flow rate, poor repeatability, slow adsorption - desorption rate and easy blockage of the imprinting sites by biomacromolecules.

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In order to improve adsorption-desorption rate, surface imprinting technique was developed, especially magnetic molecularly imprinted layer on the surface of the magnetic nanoparticles for magnetic dispersion solid-phase extraction (MD-SPE) has received increasing attention.[11-13] Compared to ordinary SPE techniques, the advantages of the MD-SPE are obvious, such as its simplicity, short extraction time, low consumption of organic solvents, easy separation of magnetic particles from complex samples.[13] Therefore, magnetic molecularly imprinted polymers have attracted considerable attention in sample preparation.[11-13] However, study of molecularly imprinted magnetic microsphere (MIMM) for selective extraction of florfenicol was not found in the literature.

In the process of sample pretreatment, biomacromolecules (such as proteins and lipids) in complex samples are accumulated on the particle surface and blocked the adsorption sites, which are still the bottleneck problem for the separation efficiency. The restricted-access materials (RAMs) with high purification efficiency have aroused great attention.[14] The RAMs generally possess dual surface configurations, the inner layer is accessible only to small molecules and has the ability for their retention and separation; the outer surface employs both size exclusion and hydrophilic interactions to prevent large biomolecules from accessing the inner surface.[14]

In order to obtain better purification and selective enrichment in complicated samples, the restricted access media-molecularly imprinted polymer (RAM-MIP) was firstly prepared by multi-step swelling and thermal polymerization method, and then the hydrophilic monomers used for hydrophilic surface modification.[15] In order to prepare uniform-sized RAM-MIP, the internal molecularly imprinted polymer layer and the external hydrophilic structures were grafted on the surface of silica microspheres by initiator-transfer agent-terminator (iniferter) technique, which not only has the selectivity for the template and its analogue, but also has the ability of exclusion for bovine serum albumin.[16]

The iniferter polymerization in the solution can be minimized because the polymeric chain propagation takes place via the active radical attached to the silica surface, while the dormant radical is in solution. The surface grafted MIP has higher separation efficiency compared with totally porous MIP material because more homogenous thin polymeric film can be formed with less mass transfer resistance.[17] Meanwhile, block polymer with different properties for different polymer layers can be grafted and the polymer layer thickness or polymeric chain length is more controllable with iniferter technique. Moreover, composite materials with different platforms can be prepared with this method. Compared with the conventional radical polymerization, the polymerization process can be well controlled by iniferter due to the avoidance of the adverse reactions such as radical coupling or disproportionation action.[18]

In the present study, a facile and highly efficient approach to prepare restricted access media-molecularly imprinted magnetic microsphere (RAM-MIMM) is based on photo-iniferter technique for selective enrichment, purification and fast separation of trace FF from the milk sample. Firstly, silica gel was coated on the surface of magnetic microsphere (Fe_3O_4@SiO_2) and magnetic iniferter was synthesized through layer-by-layer grafting from the surface of Fe_3O_4@SiO_2. Subsequently the molecularly imprinted magnetic microsphere (MIMM) was prepared by this surface-initiated (grafting from) living radical technique using FF, itaconic acid (ITA) and ethylene dimethacrylate (EDMA). Finally, the hydrophilic surface molecularly imprinted magnetic microsphere was synthesized by grafting glycidyl methacrylate (GMA) and ring-opening reaction. The RAM-MIMMs were employed as sorbent for fast clean-up and selective enrichment FF from milk samples based on surface-initiated photoiniferter-mediated polymerization.

Experimental

Chemicals and materials

Ferric chloride ($FeCl_3 \cdot 6H_2O$), anhydrous sodium acetate, tetraethoxysilane (TEOS) and perchloric acid were purchased from Tianjin Tianda Chemicals Corporation (Tianjin, China). Glycol, florfenicol (FF), chloramphenicol (CAP) and thiamphenicol (TAP) were purchased from Tianjin Huadong Chemical Reagent Co. (Tianjin, China). Itaconic acid (ITA) and coomassie brilliant blue G-250 were purchased from Beijing Chemical Reagent Co. (Beijing, China). 3-aminopropyltriethoxysilane (APTES), ethylene dimethacrylate (EDMA), 4-(chloromethyl)benzoyl chloride, sodium N,N-diethyldithiocarbamate trihydrate (DDCT) and glycidyl methacrylate (GMA) were purchased from Shanghai Bangcheng Chemical Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) and lysozyme (LZM) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). All the chemicals used were of the analytical or the HPLC grade. Ultrapure water is used throughout the experiments. Samples for HPLC analysis were filtered through a 0.45μm membrane filter.

Instruments and analytical conditions

HPLC analysis was performed using a SHIMADZU LC-20AT pump and a SPD-20A UV detector set at 224 nm. All separations were carried out on a Venusil XBP C18 column (250 × 4.6 mm, 5 μm) with a flow rate was 0.8 mL min^{-1} at 25 °C. The mobile phase was composed of methanol and ultrapure water (40:60, v/v) and the aliquots of 10 μL were injected into the column and the chromatograms were recorded. The concentrations of the FF before and after the adsorption were recorded by a T6 UV-Vis spectrophotometer (Purkinje General, China).

Preparation of RAM-MIMMs

The procedure for the synthesis of RAM-MIMMs is illustrated in the Fig. 1. A representative preparation procedure is as follows.

Preparation of the magnetic Fe_3O_4 nanoparticles. The magnetic Fe_3O_4 nanoparticles were synthesized by hydrothermal method. $FeCl_3 \cdot 6H_2O$ (2.7 g) was dispersed in glycol (80 mL) with vigorous mechanical stirring. When the solution was entirely transparent, anhydrous sodium acetate (7.2 g) was added into the solution with

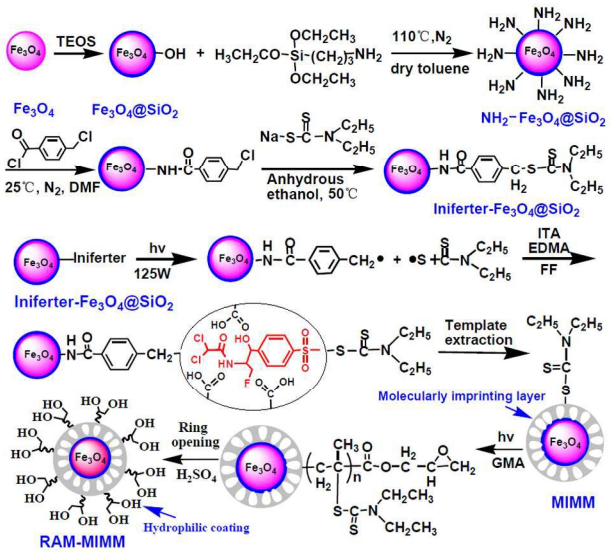

Fig. 1 Preparation protocol of the RAM-MIMMs including the iniferter bonded Fe_3O_4@SiO_2, surface grafting of the molecularly imprinted polymer layer and the hydrophilic layer.

vigorous stirring. The solution was sealed in a teflon-lined autoclave and placed in an oven to be heated at 200 °C for 10 h. The magnetic Fe_3O_4 nanoparticles were separated by a magnet and the supernatant was decanted. The black precipitate was washed with ultrapure water and ethanol three times to remove the solvent effectively, and then the product was dried under vacuum at 60 °C for 12 h.

Preparation of the Fe_3O_4@SiO_2 microspheres. The silica coated magnetic Fe_3O_4 microspheres were prepared with a sol-gel approach. Fe_3O_4 magnetic nanoparticles (1.0 g) were dispersed in a mixture of ethanol (90 mL), ultrapure water (30 mL) and concentrated ammonia (2.5 mL) under ultrasonication for 5 min. Then, a mixture of 1 mL of TEOS and 30 mL of ethanol were added to the above solution drop by drop under mechanical stirring. The reaction was carried out at room temperature under stirring for 12 h. Finally, the synthesized Fe_3O_4@SiO_2 nanoparticles were obtained by the magnetic separation, washed with ultrapure water and ethanol for 4 times thoroughly, and dried in the vacuum at 60 °C for 12 h.

Preparation of iniferter bonded Fe_3O_4@SiO_2 microspheres. The preparation of iniferter bonded Fe_3O_4@SiO_2 involves three steps (Fig. 1). In the first step, 1.0 g of Fe_3O_4@SiO_2 microspheres dispersed in 100 mL of anhydrous toluene under ultrasonication for 5 min, and 10 mL of APTES was added to the above mixture with vigorous stirring under nitrogen. In nitrogen atmosphere, the solution was stirred and refluxed for 12 h. The product (Si-I) was collected and washed with ethanol several times, then dried under vacuum at 40 °C for 12 h. In the second step, the Si-I particles were suspended in 90 mL dry DMF containing 0.4 mL pyridine. The 4-(chloromethyl)benzoyl chloride (3.7 g) dissolved in 30 mL DMF was added dropwise and the reaction was performed at 25 °C for 18 h. The Si-II particles were obtained and washed with acetone, then dried under vacuum at 50 °C for 12 h. In the third step, Si-II particles were suspended in 90 mL anhydrous ethanol and reacted with 3.0 g DDCT (dissolved in 30 mL ethanol). The reaction was performed under stirring for 8 h at 50 °C. The iniferter bonded magnetic silica (iniferter-M) was obtained and washed with distilled water and ethanol several times with the help of an external magnet, then dried under vacuum at 60 °C for 12 h.

Preparation of FF- molecularly imprinted magnetic microspheres. The molecularly imprinted magnetic microspheres (MIMMs) were prepared by a photo-grafting surface imprinting technique (Fig. 2). 0.5 g of the iniferter-M particles were mixed with a solution containing FF (0.36 g, 1 mmol), ITA (1.04 g, 8 mmol), EDMA (7.93 g, 40 mmol) and anhydrous ethanol (40 mL). The mixture was degassed with nitrogen for 10 min. Then the flask was sealed and stirred. Polymerization was initiated by UV light from a high pressure mercury lamp (125 W) at a distance of 10 cm. The reaction temperature was controlled by the ice-water bath and the reaction for 4 h. After the reaction, to remove FF, the polymers were washed with 40 mL of mixture of methanol and acetic acid (90:10, v/v) under stirring for 6 h. Then the product was collected by an external magnetic field, washed out the residue of acetic acid with methanol, and then dried under vacuum at 50 °C for 12 h.

The non-imprinted magnetic microspheres (NIMMs) corresponding to one MIMMs was synthesized for comparison. In the NIMMs synthesis, the reactant composition was the same as that in the MIMMs synthesis except the absence of the template. Because without template molecule, undesired solution polymerization appeared when the irradiation distance and time were the same as that for MIMMs, the irradiation distance was change to 20 cm and the reaction time was changed to 2 × 1 h. The material was filtered after the first 1 h reaction and the same reactant was added for the second time reaction. With this procedure, the obtained NIMMs has the same carbon content as MIMMs to ensure that the NIMMs and MIMMs have the similar affinity resulting from the poly(ITA-co-EDMA) structure (non-specific interaction).

Preparation of restricted access media - molecularly imprinted magnetic microspheres (RAM-MIMMs). Grafting of poly(GMA) chain on the MIMMs surface was performed via living radical polymerization in a lab-made glass flask with water jacket. MIMMs (0.5 g), GMA (30.2 mmol) and cyclohexanone (50 mL) were added into the flask. The reaction was initiated by UV irradiation from a high pressure mercury lamp at a distance of 10 cm. The polymerization was carried out with stirring at 40 °C under N_2 protection and the reaction for 6 h. After the reaction, the particles were washed with acetone and hydrolyzed with 0.1 mol L^{-1} of sulfuric acid at 60 °C for 8 h, the RAM-MIMMs were obtained. The restricted access media - non-imprinted magnetic microspheres (RAM-NIMMs) were also prepared using an identical procedure without FF.

Rebinding test and selectivity evaluation

50 mg of the magnetic microspheres (RAM-MIMMs, MIMMs or NIMMs) were respectively dispersed into 10 mL of various concentrations (0.05-0.5 mg mL^{-1}) of FF solutions. All of the mixtures were properly sealed and incubated under agitation in a horizontal shaker for 3 h at room temperature. Afterwards, the magnetic microspheres were isolated from the mixture by an external magnetic field and the equilibrium concentration of FF in the supernatant was determined by UV-Vis analysis. The

equilibrium adsorption capacity (Q) was calculated by using the following equation: $Q=V(Co-Ce)/m$, where V is the volume of solution (mL); m is the quality of the magnetic microspheres; Co and Ce represent the initial and equilibrium concentrations of FF in solution, respectively.

The selectivity of RAM-MIMMs was investigated with FF and structurally analogous compounds CAP and TAP at the 0.5 mg mL^{-1} level. The same procedure was performed for the RAM-NIMMs.

Exclusion protein ability evaluation

There are two kinds of method in common use. One is coomassie brilliant blue G-250 method, and the other one is ultraviolet-visible absorption method.

The first method is based on the protein interact with coomassie brilliant blue reagent. Specific steps are as follows: 50 mg of the magnetic microspheres (RAM-MIMMs, MIMMs) were respectively dispersed into 5 mL 0.5 mg mL^{-1} of bovine serum albumin (BSA) solutions, and under agitation in a horizontal shaker for 3 h at room temperature. Afterwards, the magnetic microspheres were isolated from the mixture by an external magnetic field. The concentration of BSA in the supernatant was detected via coomassie brilliant blue G-250 method. After adsorption, 0.1 mL supernatant with 5 mL of coomassie brilliant blue reagent were shaken for 5 min, and measured the absorbance values in the 595 nm. Then it could find out the corresponding concentration from the BSA standard curve. The equilibrium adsorption capacity (Q) was calculated by using the following equation: $Q=V(Co-Ce)/m$, where V is the volume of solution (mL); m is the quality of the magnetic microspheres; Co and Ce represent the initial and equilibrium concentrations of protein in solution, respectively.

Standard curve: 0.1 mL of various concentration (0, 0.1 0.2, 0.3, 0.4, 0.5 mg mL^{-1}) standard of BSA solutions, then added 5 mL of coomassie brilliant blue reagent. The mixture shocked 5 min, and measured the absorbance values in the 595 nm. The standard curve was established which the concentration of protein standard solution as the abscissa, and the absorbance of the standard solution as the ordinate. And the determination steps of lysozyme were as above.

The second method is UV-Vis absorption method. The characteristics of RAM-MIMMs exclusion protein were tested by UV-Vis spectrophotometer. In order to ensure that the measured two kinds of the original solution did not need to be diluted and the absorbance was almost same, the concentration of FF and BSA is different. Specific steps are as follows: firstly, the matrix solution of 10 μg mL^{-1} FF solution and 500 μg mL^{-1} BSA solution was tested by UV-Vis spectrophotometer. The UV absorption spectra were obtained, where FF and BSA respectively in 224 nm and 280 nm have the maximum absorption respectively. Secondly, the mixture of FF solution (10 μg mL^{-1}) and BSA solution (500 μg mL^{-1}) was analyzed by spectrophotometry. Thirdly, 10 mL of the mixture solution added into a conical flask, and 0.5 mL of perchloric acid (5%, v/v) and 20 mL ethyl acetate were also added to the above conical flask respectively. After being shaken for 1 min and centrifuged at 5000 rpm for 10 min. The supernatant was collected. The precipitate was extracted twice with 20 mL of ethyl acetate. The supernatant and eluate were merged, and evaporated to dryness with rotary evaporator at 40 ^{o}C. Subsequently, the residues were redissolved in 10 mL 50% acetonitrile-water and the solution was adjusted the pH value to 8.0, and the solution was analysed by spectrophotometer. Lastly, 50 mg of RAM-MIMMs was put in a test tube and activated in turn by 3.0 mL of water and 3.0 mL of methanol. The RAM-MIMMs were separated with a magnet, and then the above processed sample was added in this test tube. After the mixture was agitated for 25 min with a mechanic stirrer at room temperature, the supernatant was decanted. Subsequently, the RAM-MIMMs were washed with 2.0 mL of water with 1.0% acetic acid. Finally, FF was eluted from the RAM-MIMMs with 2.0 mL of 30% acetonitrile-water. The eluting solvent was tested by UV-Vis spectrophotometer. The whole process was conducted under the best condition of magnetic dispersion extraction.

The enrichment of the RAM-MIMMs

50 mg of RAM-MIMMs were respectively applied to 0.5 μg mL^{-1} of 10 mL mixture standard solution (FF, CAP and TAP) that had been adjusted to pH 8.0. The mixture was agitated for 25 min with a mechanic stirrer. Subsequently, the magnetic microspheres were isolated from the mixture by an external magnetic field. The analytes were desorbed from the isolated particles with 2 mL 30% acetonitrile-water under sonication for 2 min. Finally, the eluate was evaporated at 50 ^{o}C, and then the residue was dissolved in 1 mL of mobile phase.

The magnetic dispersion extraction of FF from milk samples

All milk samples were pretreated before analysis by the following procedure. 10 g of milk sample was added into a conical flask, and 0.5 mL of perchloric acid (5%, v/v) and 20 mL ethyl acetate were also added to the above conical flask respectively. After being shaken for 1 min and centrifuged at 5000 rpm for 10 min. The supernatant was collected. The precipitate was extracted twice with 20 mL of ethyl acetate. The supernatant and eluate were merged, and evaporated to dryness with rotary evaporator at 40 ^{o}C. Subsequently, the residues were redissolved in 50% acetonitrile-water and the solution was adjusted the pH value to 8.0. 50 mg of RAM-MIMMs was put in a test tube and activated in turn by 3.0 mL of water and 3.0 mL of methanol. The RAM-MIMMs were separated with a magnet, and then the above processed sample was added in this test tube. After the mixture was agitated for 25 min with a mechanic stirrer at room temperature, the supernatant was decanted. Subsequently, the RAM-MIMMs were washed with 2.0 mL of water with 1.0% acetic acid. Finally, FF was eluted from the RAM-MIMMs with 2.0 mL of 30% acetonitrile-water and then evaporated to dryness at 50 ^{o}C under nitrogen. The residues were redissolved in 1.0 mL of mobile phase for HPLC analysis. The milk was spiked with FF at three different levels with 100, 200 and 400 μg kg^{-1}, and each experiment was carried out in triplicate.

Results and discussion

Characterization of RAM-MIMMs

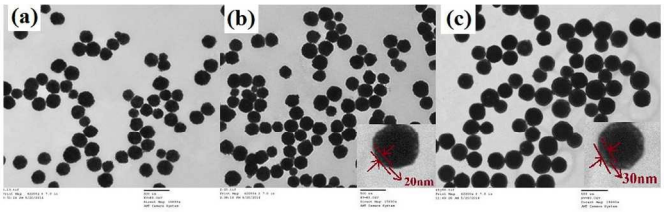

Fig. 2 TEM images of (a) Fe_3O_4, (b) MIMMs and (c) RAM-MIMMs.

Morphological characterization. TEM was utilized to observe the morphological features of Fe_3O_4, MIMMs and RAM-MIMMs. Fe_3O_4 nanoparticles exhibited the spherical shape with the mean diameter of 230 nm (Fig. 2a,). After MIMMs and RAM-MIMMs grafting, these nanoparticles still remained the fine spherical morphology (Fig. 2b-c). The hydrophilicity of the external surface was modified by epoxide ring-opening with poly(GMA). The results showed that a satisfactory RAM-MIMM was obtained. From the TEM image of MIMMs (Fig. 2b) and RAM-MIMMs (Fig. 2c), it was obvious that MIMMs and RAM-MIMMs were regular spheres with a mean diameter of 250 and 260 nm respectively and the surface of synthesized RAM-MIMMs was rough. Fig. 2c shows the distinct core-shell structure of the hydrophilic imprinted film-coated silica nanoparticles with 30 nm thin layer. This image suggests that core-shell nanoparticles with more regular morphological features were prepared through a step-by-step grafting procedure. This image also reveals that the grafting reaction process did not significantly result in the agglomeration and change in size of particles, which can be attributed to the fact that the reaction occurred only on the particle surface.

Thermogravimetric analysis. The thermogravimetric analysis was performed to further estimate the grafting yield of imprinted polymer layer on RAM-MIMMs nanoparticles. Figure 3 reveals the TGA curves of Fe_3O_4, Fe_3O_4@SiO_2, iniferter-M, MIMMs and RAM-MIMMs in the temperature range of 50-800 ℃. Clearly, during the successive heating process, the mass changes are very slight for Fe_3O_4 and Fe_3O_4@SiO_2. At 800 ℃, the mass losses of $Fe_3O_4$and Fe_3O_4@SiO_2 are 1.2 and 2.5 wt %, respectively, indicating the stability of these nanoparticles. For the iniferter-M, a mass loss of 4.2 wt % was found after continuous heating (Fig. 3c), indicating

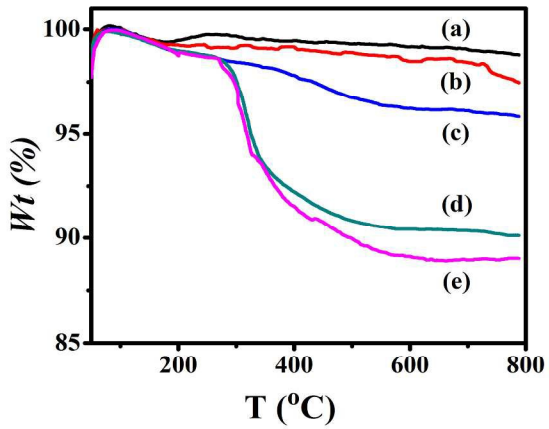

Fig. 3 TGA curves of (a) Fe_3O_4, (b) Fe_3O_4@SiO_2, (c) iniferter-M, (d) MIMMs and (e) RAM-MIMMs.

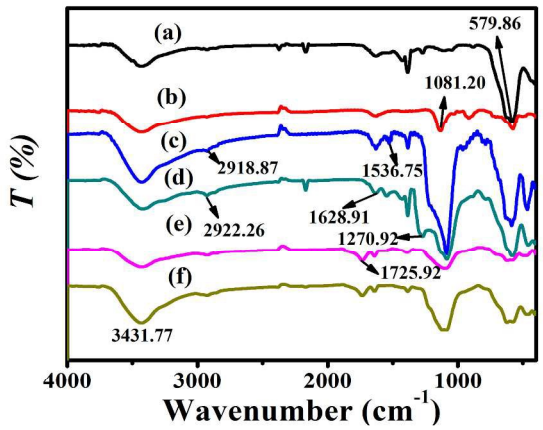

Fig. 4 FI-IR spectra of (a) Fe_3O_4, (b) Fe_3O_4@SiO_2, (c) amino modified Fe_3O_4@SiO_2, (d) iniferter-M, (e) MIMMs and (f) RAM-MIMMs.

the successful modification of the iniferter agent. In contrast, dramatic mass change was detected for MIMMs and RAM-MIMMs. The MIMMs and RAM-MIMMs polymer layer began to thermally decompose at about 270 °C, accompanied by a significant mass loss. The mass loss continued up to about 550 °C, after that, the mass kept relatively constant. The total mass loss of MIMMs and RAM-MIMMs reaches to 10.0 and 11.0 wt % respectively until 800 °C. The results revealed the grafting of hydrophilic imprinted polymer on the surface of Fe_3O_4@SiO_2 was successful and effective.

Characteristic of the FT-IR spectra. To ascertain the presence of the RAM-MIMMs, FT-IR spectra were obtained from Fe_3O_4, Fe_3O_4@SiO_2, amino-modified, MIMMs and RAM-MIMMs. As shown in Fig. 4a, the observed features around 579.86 cm^{-1} attributed to the Fe_3O_4 characteristic peak. Fig. 4b showed the bands around 1081.20 cm^{-1} resulted from Si-O-Si stretching vibrations. In Fig. 4c, the adsorption observed around 1536.75 cm^{-1} and 2918.87 cm^{-1} indicated the existence of amino-groups in the modified silica nanoparticles, and in Fig. 4d we could see the band at 1270.92 cm^{-1} was attributed to thiocarbonyl group of the iniferter, suggesting that the iniferter was modified onto Fe_3O_4 micropheres. In comparison with Fig. 4d, Fig. 4e showed the absorption peak at 1725.92 cm^{-1} was assigned to ester carbonyl stretching vibrations, which indicated ITA and/or EDMA were bonded in the material. In addition, compared with MIMMs, the aliphatic hydroxyl adsorption peak at 3431.77 cm^{-1} of the RAM-MIMMs was obviously increased in Fig. 4f.

Optmization of magnetic dispersion extraction conditions

Effect of sample pH. The effectiveness of the adsorption of an analyte onto the sorbent depends significantly on sample pH. Taking into account the presence of OH and NH groups in the FF molecule and the pKa value of FF (9.03), pH may influences the existing form of the FF, so in high acidic solutions, protonation of functional groups in FF resulted to decrease in the specific recognition of MIMMs to analyte. For this, the effect of sample pH on the adsorption of FF was studied over the pH range 2.0-10.0 using 50 mg of magnetic microspheres in 10 mL of the solution containing 0.5 mg mL^{-1} of FF. The results were shown in Fig. 5a. The results

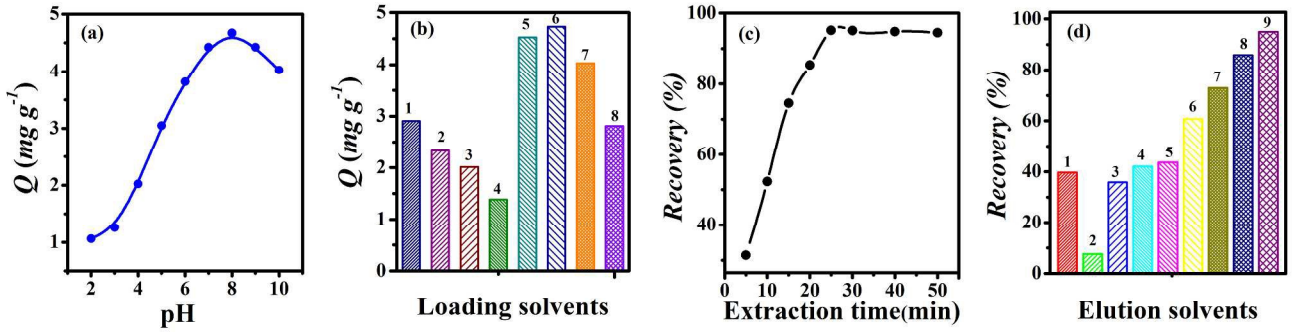

Fig. 5 The effects of (a) sample pH, (b) loading solvents, (c) extraction time and (d) elution condition on the extraction efficiencies were studied. The loading solvents: 1. methanol; 2.50% methanol-water; 3. 25% methanol-water; 4. 5% methanol water; 5. acetonitrile; 6. 50% acetonitrile-water; 3. 25% acetonitrile-water; 4. 5% acetonitrile-water. The elution solvents: 1. water; 2. 1% acetic acid-water; 3. 2% acetic acid-water; 4. 5% methanol-water; 5. 5% ethanol-water; 6. 5% acetonitrile-water; 7. 10% acetonitrile-water; 8. 20% acetonitrile-water; 9. 30% acetonitrile-water.

showed a maximum sorption efficiency of FF at pH 8.0, therefore for further experiments, all samples adjusted to this pH value.

Effect of loading solvents. The effectiveness of the adsorption of an analyte onto the sorbent depends significantly on sample solubility. We investigated different loading solvents which including different percentage (5%, 25%, 50%, 100%) of acetonitrile present in the purified water and different percentage (5%, 25%, 50%, 100%) of methanol present in the purified water. Fig. 5b indicated that 50% acetonitrile-water solution was sufficient to achieve high adsorption capacity.

Effect of extraction time. The extraction procedure includes three steps: adsorption, washing and elution. The total time required for extraction is a key factor in the efficiency of the assay. As described for the magnetic dispersion extraction procedure, the interaction between the FF and the sorbent was promoted by agitation. The effect of the adsorption time was studied by varying the stirring time (0-60 min). Fig. 5c indicated that 25 min was sufficient to achieve complete recovery. After the adsorption stage, the washing and elution steps took about 5 min. The whole extraction procedure could be accomplished within 30 min, which was superior to conventional SPE, solid-phase microextraction and stir bar sorptive extraction.

Effect of elute solvents. In order to enhance the selectivity of RAM-MIMMs and decrease the matrix interference, the washing conditions were optimized. It is well known that compounds could be retained on the imprinted sorbents due to both specific and non-specific interactions. Thus, a washing solution with moderate elution strength was used to damage the nonspecific interactions and to let the target analyte be retained by specific interactions. As seen from Fig. 5d, for the recovery of strongly bound FF, the cartridge was eluted with 5 mL of different solvents including various aqueous media including acetic acid (1% and 2%, v/v), methanol (5%, v/v), ethanol (5% v/v), acetonitrile (5%, 10%, 20%, 30%, v/v), and purified water were assessed. The results showed that when acetic acid was added to purified water at concentrations 1% was used for eluent, the recovery reaches its lowest point. So, purified water containing 1% acetic acid was selected as the washing solvent. For the recovery of strongly bound FF, acetonitrile-water solution (30%, v/v) provided the best results.

Rebinding test and selectivity evaluation

The adsorption capacity was an important factor, because it determined how much imprinted sorbent was required to quantitatively concentrate the analytes from a given solution. As can be seen in Fig. 6, the adsorption capacity increased as initial FF concentration was increased until the stable values were obtained. The adsorption capacities of RAM-MIMMs and MIMMs for FF were approximately equal to 5.0 mg g^{-1}, and the adsorption capacities of RAM-NIMMs for FF were calculated as 2.6 mg g^{-1}. The adsorption capacity of RAM-MIMMs was about 1.9 times than NIMMs. In general, the RAM-MIMMs possesses both specific and nonspecific binding sites, while the RAM-NIMMs only has nonspecific binding sites, which enables the RAM-MIMMs to take up more FF than the RAM-NIMMs. The template molecules are first adsorbed mainly to the nonspecific sites rather than the specific sites. After most of the nonspecific sites have been occupied, the specific sites began to get occupied. This is why the adsorption capacities of the RAM-MIMMs and RAM-NIMMs are similar at low concentrations. What's more, in our experiments, it was found that adsorption capacity of MIMMs slightly higher than the RAM-MIMMs, which may be because the grafting hydrophilic group affected the template molecules into the imprinting sites.

The selectivity of RAM-MIMMs was investigated by the dynamic competitive adsorption experiments according to the previous extraction conditions for FF and its structural analogues (CAP and TAP). Distribution coefficient (K_D), selectivity coefficient (k) and relative selectivity coefficient (k') was obtained and the results were

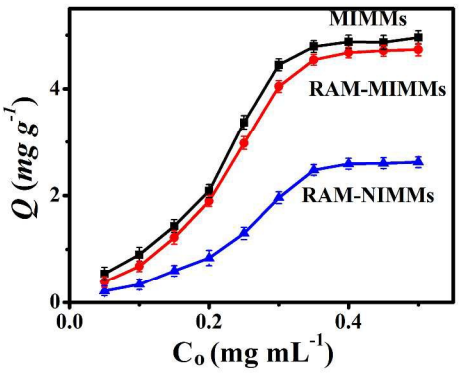

Fig. 6 Binding isoterm of the binding of FF onto RAM-MIMMs, MIMMs and RAM-NIMMs.

Table1 Competitive adsorption of FF, CAP and TAP by the RAM-MIMM (A) and the RAM-NIMM (B)

Analyte	C_0	C_f		K_D/ mL g^{-1}		k		k'
		A	B	A	B	A	B	
FF	500	479.6	490.4	8.51	3.92			
CAP	500	482.5	486.3	7.25	5.63	1.17	0.70	1.68
TAP	500	483.1	487.2	7.00	5.25	1.22	0.75	1.63

K_D= (C_0 - C_f) V/m C_f, where C_0 and C_f (mg mL^{-1}) represented the initial and final concentration; $k = K_{D(FF)}/K_{D(CAP)}$;$k' = k_{RAM\text{-}MIMM}/k_{RAM\text{-}NIMM}$.

listed in Table 1. K_D= (C_0- C_f) V/m C_f, where C_0 and C_f (mg mL^{-1}) represented the initial and final concentration. The selectivity coefficient of the sorbent suggested the otherness of two substances adsorbed by one sorbent, $k = K_{D(FF)}/K_{D(CAP)}$; the relative selectivity coefficient suggested the otherness of two sorbents, $k' = k_{RAM\text{-}MIMM}/k_{RAM\text{-}NIMM}$. As shown in Table 1, FF, CAP and TAP had the similar K_D on the RAM-NIMMs, but the RAM-MIMMs showed $K_{D(FF)}$ was greater than similar compounds. The k value of RAM-MIMMs was larger than that of the RAM-NIMMs, which showed that the RAM-MIMMs had high selectivity for FF over the analogues. The relative selectivity coefficient was 1.68 and 1.63 for CAP and TAP, respectively, which showed the high selectivity of the RAM-MIMMs than the RAM-NIMMs. The superior rebinding ability proved that the target molecules were not simply adsorbed on the material surface, but selectively trapped in the imprinting cavities through hydrogen bonding and hydrophobic interactions.

Exclusion protein capability evaluation

The capability of exclusion protein between the RAM-MIMMs and MIMMs was evaluated by adsorbing of bovine serum albumin (BSA) and lysozyme (LZM) solution respectively with coomassie brilliant blue G-250. From Fig. 7A, despite the difference in molecule weight of two proteins, their adsorption quantity was roughly the same. The smaller the Q value was, the stronger exclusion protein ability was. Thus, this indicated that macromolecules are excluded and interact only with the outer surface of the RAM-MIMMs, which minimizes the adsorption of matrix proteins.

Through the UV absorption method, we evaluated protein exclusion ability. In Fig. 7c, the absorbance of the protein decreased,

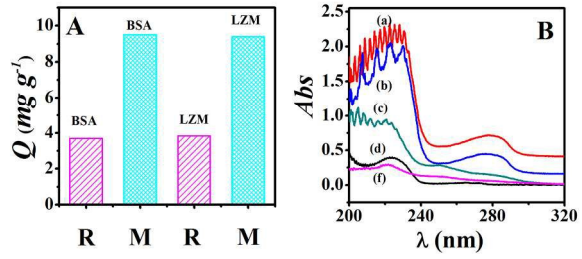

Fig. 7 The exclusion protein ability of RAM-MIMMs.

A:The exclusion protein ability of RAM-MIMMs and MIMMs with coomassie R-250 method (e.g., R = RAM-MIMMs, M = MIMMs) .

B: The exclusion protein ability of RAM-MIMMs with UV absorption method. The UV spectrogram of (a) 500 μg mL^{-1} BSA solution; (b) the mixture of 10 μg mL^{-1} FF solution and 500 μg mL^{-1} BSA solution; (c) the mixture solution after pretreatment; (d) 10 μg mL^{-1} FF solution; (f) the mixture solution after clean-up.

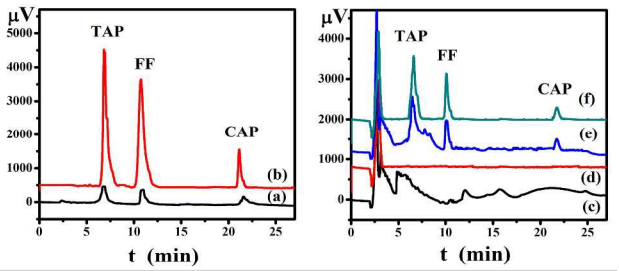

Fig. 8 Enrichment and sample clean-up chromatograms were obtained from the milk samples. Chromatograms of (a) the mixture standard solution (0.5 μg mL^{-1}); (b) the eluate of 10 mL of the mixture standard solution through MDE with the 50 mg of RAM-MIMMs; (c) Blank milk (non-spiked); (d) blank milk with a clean-up of the 50 mg of RAM-MIMMs; (e) a spiked milk (100 μg kg^{-1}); (f) spiked milk (100 μg kg^{-1}) with a clean-up of 50 mg of RAM-MIMMs. The mobile phase was methanol/water solution (40:60, v/v). The flow rate was 0.8 mL·min^{-1}. The analytes were detected at 224 nm.

but it could not be completely removed. As shown in Fig. 7f, under the optimization conditions of the magnetic dispersion extraction, BSA peak was essentially rule out and FF peak was only existed. So the method is feasible.

Enrichment factor of MDE and sample clean-up

The amount of adsorbent was an important factor which indicated the enrichment ability of RAM-MIMMs for the target analyte at a very low concentration. 50 mg of RAM-MIMMs was applied to magnetic dispersion extraction (MDE) of 10 mL of the mixture standard solution (0.5 μg mL^{-1} of TAP, FF and CAP). The extraction procedure has been described in experimental section. The chromatograms of the mixture standard solution and the mixture standard solution through MDE were respectively shown in Fig. 8a and Fig. 8b. The enrichment factors were 8.67, 8.63 and 6.58 for TAP, FF, and CAP, respectively.

In order to investigate the potential of the RAM-MIMMs for the selective entrapment of target analyte from complex milk samples, satisfactory sample clean-up was achieved by magnetic dispersion extraction (MDE). Fig. 8 revealed the chromatograms obtained for the blank milk sample (Fig. 8c), the blank milk after MDE (Fig. 8d), the spiked milk (Fig. 8e) and the spiked milk after MDE (Fig. 8f). Figure 8c and Figure 8d showed chromatograms of the blank milk by direct injection analysis and the blank milk after MDE with the 50 mg of RAM-MIMMs. The comparison of them indicated that the proposed method had obtained the good clean-up effect. Then, as shown in Fig. 8e, the milk sample spiked with FF and its analogues (TAP and CAP) could be found in HPLC analysis by direct injection, but the quantification was difficult due to the very weak signal and the interference from the sample matrix components. When the spiked sample was treated with RAM-MIMMs and analyzed, the interfering peak of the sample matrix components reduced and FF was concentrated (Fig. 8f). The results indicated that the method provided satisfactory clean-up of milk sample.

Determination of FF in milk samples

Under the optimized conditions, the RAM-MIMMs were applied to magnetic dispersion extraction of FF from milk sample. The accuracy of the method was estimated by determining milk sample

Table 2 Average recoveries (R), relative standard deviations (RSDs, $n = 3$), limit of detection (LOD) and limit of quantitation (LOQ) of BPA and its analogues were obtained after MDE of the spiked milk samples ($n = 3$).

Analyte	Spiked level (μg kg^{-1})	Recovery (%)	RSD (%)	LOD[a] (μg kg^{-1})	LOQ[b] (μg kg^{-1})
TAP	100	86.2	2.95	4.40	14.66
	200	89.7	3.31		
	400	91.6	3.44		
FF	100	89.2	3.49	6.38	21.28
	200	92.2	3.98		
	400	90.2	4.19		
CAP	100	85.4	4.97	18.31	61.02
	200	94.7	4.48		
	400	90.7	3.51		

[a] LOD calculated as 3 times the signal-to-noise ratio;
[b] LOQ calculated as 10 times the signal-to-noise ratio.

spiked with FF, TAP and CAP at three different concentration levels (100, 200 and 400 μg kg^{-1}). The results were shown in Table 2. The average recoveries of FF, TAP and CAP from spiked milk sample were in the range of 85.4-94.7% with relative standard deviations (RSDs) of 2.95-4.97%. The limits of determination (LOD, S/N = 3) and the limits of quantitation (LOQ, S/N = 10) of the milk samples were 4.40 and 14.66 μg kg^{-1} for TAP, 6.38 and 21.28 μg kg^{-1} for FF, 18.31 and 61.02 μg kg^{-1} for CAP. The LOQ could meet the requirement of specific migration limits determination. The proposed method has a lower LOD and better clean-up effect than the SPME method and the method of magnetic molecularly imprinted polymer, respectively. So the present sample preparation procedure is simple and could be effective for the analysis of environmental, food and biological samples.

Conclusions

A novel restricted access media-molecularly imprinted layer grafted on surface of the magnetic silica microspheres was successfully synthesized using surface-initiated iniferter technique. Florfenicol imprinted polymer internal layer and hydrophilic external layer were immobilized on the surface of magnetic silica successfully. The material has the properties of MIP and RAM and can be used as sorbent for magnetic dispersion extraction and sample clean-up in the florfenicol analysis in milk. Good accuracy and precision were obtained, indicating that the method can be applied in the determination of chloramphenicols in milk with good reliability. This research demonstrated that the iniferter technique provides a way of synthesizing the RAM-MIMMs. The RAM-MIMMs exhibit an application potential in drug residue analysis for complex samples with high selectivity and analytical efficiency.

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Graphical abstract

Fast clean-up and selective enrichment of florfenicol in milk by restricted access media - molecularly imprinted magnetic microspheres based on surface-initiated photoiniferter - mediated polymerization

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A novel RAM-MIMM with water-compatible, exclusion biomacromolecules and selective enrichment analytes was prepared by surface-initiated iniferter technique for magnetic dispersion microextraction of trace florfenicol from milk samples.