

Preparation of Magnetic Molecularly Imprinted Polymer for Melamine and its Application in Milk Sample Analysis by HPLC

Yuxia Qi¹, Guanyu Li²,
Chanling Wei¹, Lijuan Zhao¹,
and Bolin Gong^{1,3}

Abstract

In this work, a simple and selective method was successfully developed for the determination of melamine (MEL) in milk sample using magnetic surface molecularly imprinted polymer (MMIPs) as sorbent. The MMIPs has been prepared by using MEL as template molecule, methacrylic acid (MAA) as functional monomer and Fe₃O₄@ SiO₂ as magnetic support. The polymer was characterized by means of fourier transform infrared(FT-IR) analysis, X-ray diffraction (XRD) analysis, thermogravimetric Analysis (TGA), scanning electron microscope(SEM) and vibrating sample magnetometer (VSM). The results demonstrated that an imprinted polymer layer was successfully coated onto the surface of modified Fe₃O₄ nanomaterials, resulting in a good magnetic responsibility. The UV spectrophotometry was used to demonstrate the interaction between MEL and MAA. It was found that one MEL was entrapped by two MAA molecules. Binding selectivity, adsorption capacity and the reusability for MMIPs were also evaluated in details. Results indicated the prepared MMIPs exhibited high adsorption capacity, fast binding ability and high selectivity for melamine. Detection limits for melamine were within the range of 0.15-3.00 µg/mL, which could meet the analysis of trace melamine in milk. Calibration curve was established with R of 0.9997. The limit of detection (LOD) of this new method was 15 µg/L. The obtained results revealed that the extraction recoveries of melamine from real samples were in the range of 85.6% to 104.2%. This work proposed a sensitive, rapid, reliable and convenient approach for the determination of trace melamine in complicated milk sample.

Keywords: Melamine; Magnetic molecularly imprinted polymer; Fe₃O₄@ SiO₂; Recognition character; HPLC

- 1 College of Chemistry and Chemical Engineering, Beifang University of Nationalities, Yinchuan, China
- 2 Department of Architectural Engineering, Gansu Forestry Technological College, Tianshui, China
- 3 College of Chemistry and Chemical Engineering, Ningxia University, Yinchuan, China

Corresponding author: Bolin Gong

✉ gongbolin@163.com

College of Chemistry and Chemical Engineering, Beifang University of Nationalities and College of Chemistry and Chemical Engineering, Ningxia University, Yinchuan 750021, China

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Introduction

Melamine (2,4,6-Triamino-s-triazine, MEL) is an important chemical widely used in the production of melamine resins, which are used in laminates, glues, adhesives, and plastics [1]. Because of its wide dispersive use and toxic properties [2,3], monitoring of melamine (MEL) has been giving rise to international concern. In 2008, MEL caused the death of certain infants in China, who had drunk milk containing this compound. However, milk being a very complex substrate, for the analysis of complex samples, sample preparation is a crucial and important step in the whole analytical procedure. Numerous analytical approaches for

sample preparation and simultaneous determination of MEL have also been published, including high-performance liquid chromatography (HPLC) [4-6], liquid chromatography-mass spectrometry (LC-MS) [7,8], capillary electrophoresis (CE) [9], and gas chromatography-mass spectroscopy (GS-MS) [10,11]. Most of these methods rely on time-consuming, laborious sample pretreatment procedures and the cost is higher.

The molecular imprinting technique had been already achieved rapid developments in the separation field due to its high selectivity, stability, and preparation with ease. In researches reported, most Molecularly imprinted polymers (MIPs) for MEL were prepared by bulk polymerization or precipitation polymerization [12,13].

These kinds of MIPs exhibit high selectivity but low rebinding capacity, poor site accessibility to target species and leakage of template molecules [14-16], because the imprinted polymer matrices are usually thick, and the template molecules are embedded in the matrices too deeply to be fully eluted. In order to overcome these drawbacks effectively, the surface molecular imprinting technique has been developed. With this technique, the disadvantages of traditional MIPs have been avoided to some extent. However, little attention has been paid to synthesize MIPs for MEL by the surface molecular imprinting technique. In recent years, magnetic separation technology has received considerable attention, for they can easily be separated from extraction system by the application of an external magnetic field. Chen et al. [17] and Wang et al. [18] had synthesized the MMIPs, which can be directly added into the samples and separated from the extraction system easily without additional centrifugation and filtration, simplifying the pretreatment procedures. Wang et al. [19] had synthesized the magnetic molecularly imprinted polymers (MMIPs) for the extraction of MEL from milk. But in their method, tedious solvent extraction and centrifugation were still needed, which had not taken full advantage of the magnetic materials. He et al. [20] reported a method for determination of MEL from milk using Fe_3O_4 magnetite as magnetic component. Fe_3O_4 was easily oxidation and aggregation as we known it. Therefore, Fabrication of nano-scale MMIPs with functionalized magnetic nanoparticles materials is necessary. But, as far as we know, the application of MMIPs technology to extract the MEL from milk samples with $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ as the support, carrying out the extraction and separated procedures in one step, has not been reported yet.

In this paper, a simple method was developed to improve and optimize the analysis of MEL in milk. The new kind of MMIPs was synthesized by using MEL as template molecule, and was successfully applied for the extraction of MEL from milk samples. The proposed method could achieve the extraction and enrichment steps simply by blending and stirring the MMIPs and the milk sample which was diluted with water. The method is more effective and less time-consuming. Meanwhile, high extraction recovery and the high sensitivity of this method showed great advantage than the classic method.

Experimental

Materials

Melamine (MEL), Cyanuric acid (CYA), Cyromanize (CYR), Tetraethyl orthosilicate (TEOS), methacrylic acid (MAA) Ethylene glycol dimethacrylate (EDMA) and 2,2'-Azobis(2-methylpropionitrile) (AIBN) were obtained from Aladdin Reagent (Shanghai, China). 3-methacryloylpropyl trimethoxysilane (MPS) was obtained from Shanghai Chemical Reagent Plant (Shanghai, China). Oleic acid, Ammonium acetate, Ethanol absolute, Acetic acid, Iron chloride, methanol and acetonitrile were purchased from the Tianjin chemical reagent company (Tianjin, China). All other reagents were of analytical grade. Milk samples were purchased from a local supermarket. MAA was purified prior to use via the general distillation method in vacuum under nitrogen protection to remove the polymerization inhibitor. Double distilled water

was used throughout the experimental procedures. HPLC grade acetonitrile was obtained from Sigma (St. Louis, MO, USA), All solutions used for HPLC were filtered through a nylon 0.45 μm filter before use.

Instruments

All chromatographic tests were performed using an LC-20AT chromatographic system (Shimadzu, Japan), including two pumps and a multiple-wavelength detector. Samples were injected through a Rheodyne 7725 valve. Thermo-gravimetric analyses (TGA) were carried out simultaneously using a ZRY-2P instrument (Shimadzu, Japan). TU-1810-type ultraviolet spectrophotometer (Beijing general instrument co., Ltd., China). The Fourier Transform Infrared (FT-IR) spectroscopy (Shimadzu, Japan), Magnetic properties were determined with a 7410 vibrating sample magnetometer (VSM, Lake Shore, Cryotronics Inc., USA), Dmax2200pc type X-Ray Diffraction (XRD) (Rigaku co., Japan) and JSM-7500F type Scanning electron microscope (JEOL co., Japan) were used in this experiment.

Preparation of the magnetic molecularly imprinted polymer

Preparation of the magnetic molecularly imprinted polymer was essentially using the procedure reported by Men [21] with modifications. The preparation method of MMIPs beads is schematically illustrated in **Figure 1**.

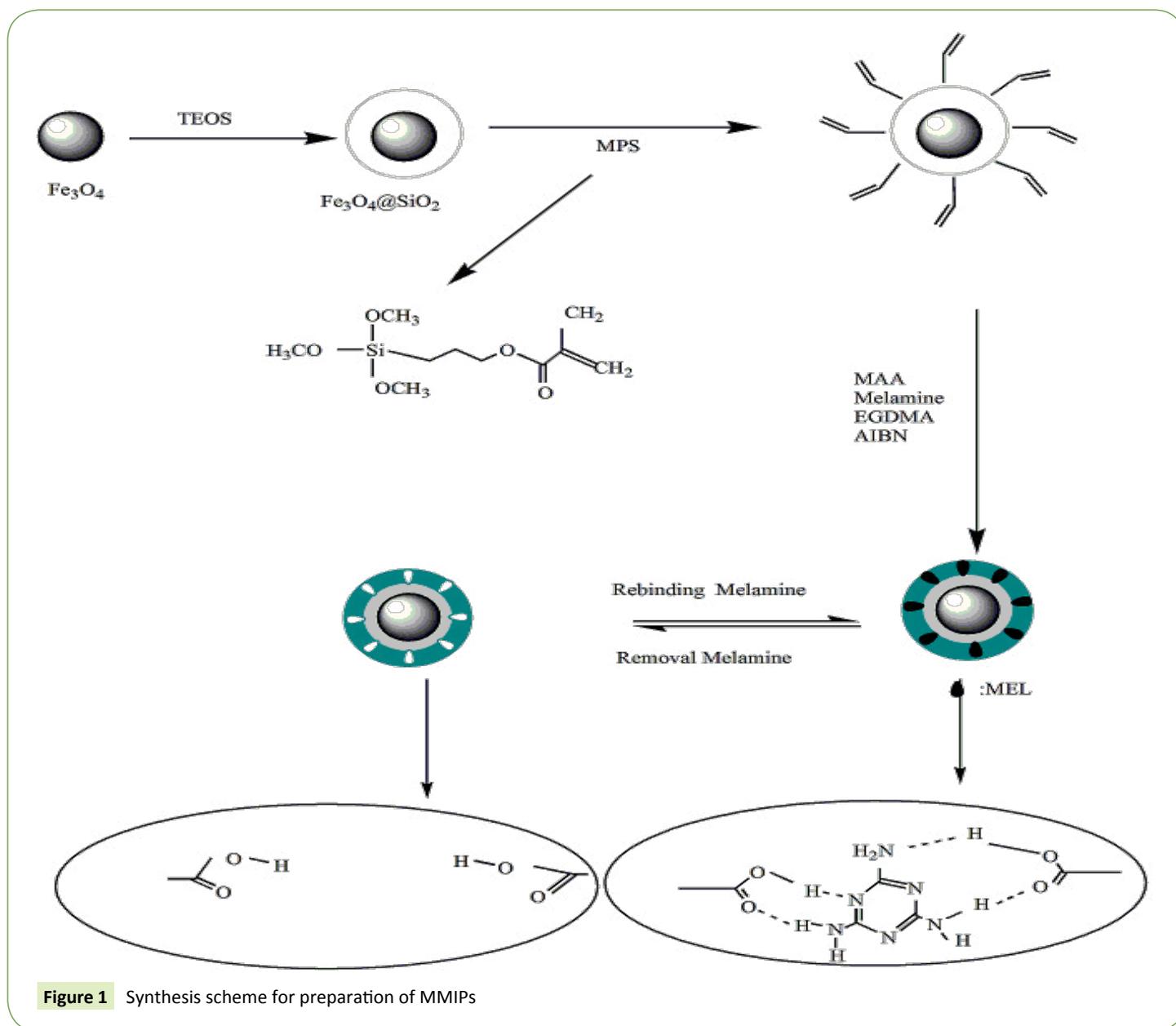
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.05 mol/L) was dissolved in 60 mL ethanol aqueous solution and heated to 50°C. Added hydroxylamine hydrochloride (0.0511 g) after 5 min ammonia to make pH>9 then oleic acid was slowly added, After 1 h, the obtained Fe_3O_4 nanoparticles were washed with distilled water and ethanol until it was neutral. The oleic acid-coated magnetic nanoparticles particles were dried under vacuum at 50°C.

$\text{Fe}_3\text{O}_4@ \text{SiO}_2$ microspheres were prepared according to the sol-gel method. Typically, Fe_3O_4 (0.1 g) was dispersed in ethanol (60 ml) and ultrapure water (10 ml) by sonication for 15 min, followed by the addition of ammonium hydroxide (1 mL) and TEOS (2 mL) sequentially. The mixture solution reacted for 24 h at the room temperature under a continuous stirring. The synthesized product was separated by a magnet and washed with ethanol and deionized water several times, and dried under vacuum at 60°C.

To prepare the $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ particles modified with 3-methacryloxypropyl trimethoxysilane (MPS), 1.0 g $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ activation microspheres were dispersed in 100 mL of mixed solvent of ethanol-water (1:1, v/v), followed by the addition of 1.5 ml of 3-methacryloxypropyl trimethoxysilane (MPS). Then, the mixture was incubated at 60°C for 24 h under nitrogen protection. The $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ microspheres modified by MPS ($\text{Fe}_3\text{O}_4@ \text{SiO}_2$ -MPS) was obtained by magnetic separation and rinsed with ethanol several times.

Then MMIPs films were synthesized as follows process. The template (melamine, 1 mmol) and functional monomer

(MAA, 4 mmol) were dispersed in ethanol-water (4:1, v/v) (20 mL) by sonication, and the mixture has been shaken for overnight.



Then, the $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ -MPS particles (0.2 g), the cross-linking monomer (EDMA, 8 mmol), and the initiator (AIBN, 0.06 g) were added to the mixture, respectively. Subsequently, the solution was deoxygenated by purging with nitrogen for 30 min, sealed and shaken at 60°C for 24 h. Upon completion, template molecule of the resulting product were removed with a mixture solvent of methanol and acetic acid (9:1, v/v) by soxhlet extraction until the eluent was free from melamine through detected by UV spectrophotometer. Finally, the MMIPs obtained were washed with methanol to remove the remaining acetic acid and dried in the vacuum.

As a reference, magnetic non-imprinted polymer (MNIPs) were also prepared using the same procedures but without adding melamine templates.

Static adsorption test

To measure the adsorption capacity of the polymer, 20 mg of

MMIPs or MNIPs sorbent added to 10 mL of 20~120 mg/L MEL mixed solution of ethanol-water (4:1,v/v) was shaken for 6 h. The resulting supernatant was measured for unbound MEL by UV spectrometry. The adsorption capacity is calculated according to the equation as follows.

$$Q = \frac{(C_i - C_f) \times V}{m} \quad (1)$$

Where Q (mg/g) was the mass of MEL adsorbed per gram of polymers, C_i (mg/L) and C_f (mg/L) are the initial and final concentrations of MEL in solution, respectively, V (L) is the total volume of the solution, and m (g) is the mass of polymers.

The saturation binding data were further processed to generate a Scatchard equation to estimate the binding properties of MMIPs and MNIPs. The Scatchard equation was as follows:

$$\frac{Q}{C_{\text{free}}} = \frac{Q_{\text{max}} - Q}{K_d} \quad (2)$$

Where Q was the amount of MEL bound to polymers at equilibrium, Q_{max} was the apparent maximum adsorption capacity, C_{free} was the free MEL concentration at equilibrium and K_d was the dissociation constant. The values of K_d and Q_{max} could be calculated from the slope and intercept of the linear curve plotted at Q/C_{free} versus Q.

Selective binding experiments

Adsorption and competitive recognition studies were performed with MEL, cyanuric acid (CYA), cyromazine (CYR) (Figure 2). The MMIPs (20 mg) was added to a flask containing 10 mL of 90 mg L-1 MEL, CYA and CYR, respectively, shaken at room temperature for set times, and then separated centrifugally.

Milk sample treatment

A portion of 10 mL milk was mixed with melamine standard solutions and was homogenized. First, protein was removed from the matrix by adding acetonitrile (20 mL) to 10 mL milk. The spiked samples were extracted by ultrasonic assisted extraction at room temperature for 30 min; the mixed sample was transferred to two 20 mL centrifuge tubes. Supernatant extract was isolated after centrifugation for 10 min at speed of 4000 rpm. The supernatant was collected to reserve.

10 mL extract into 25 mL conical flask and then 20 mg MMIPs were added. The complex was shaken for set times at room temperature. The MMIPs were obtained by magnetic separation, and then moderate amount of methanol and acetic acid (9:1, v/v) was used as the eluted reagent to extract the analytes in MMIPs. The supernatant was obtained and dried under a N₂ stream. Finally, the residues were redissolved in 1 mL of mobile phase for further HPLC analysis. The spiking concentrations for each MEL were set with three levels of 200.0, 300.0, and 500.0 µg/L, respectively.

HPLC analysis

Column chromatography conditions: Luna HILIC (250 mm × 4.6 mm, 5 microns, Phenomenex companies in the US), mobile phase: acetonitrile-10 mmol/L ammonium acetate (9:1, v/v), detection wavelength: 210 nm, injection volume : 20 µL.

Results and Discussion

MEL and MAA combining with mechanism analysis

Interactions between functional monomer and the template molecule directly affect the MMIPs recognition and adsorption performance. The molecular imprinted polymer (MIPs) which have a high selectivity and recognition to template is mainly by the interactions between the mode and intensity. The interaction between template molecule and functional monomer are stronger, MIPs is stronger affinity ability of template molecule. Therefore, the interaction between template and functional monomer molecules were studied before the preparation of MMIPs it was necessary. Figure 3a of the molar ratio of template molecule and functional monomer for a 1:0, 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8, respectively. On the ultraviolet spectrophotometer measured the absorption curve of 10 kinds of mixture and curve plotting the differential ultraviolet absorption spectrum.

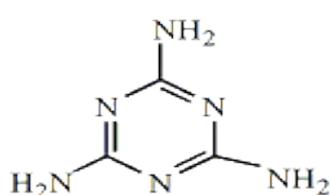
In theory, template molecule and functional monomer to form polymer, according to the following equations:

$$\frac{\Delta A}{b_0^n} = -K\Delta A + K\Delta \xi_c a_0 l \quad (3)$$

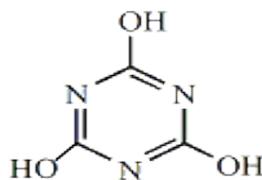
Where K was constant, ξ_c was the absorbance coefficient of template molecule and functional monomer compounds, ΔA for functional monomer and the template molecules in ethanol-aqueous solution absorbance difference before and after effect, b_0 was the concentration of functional monomer, a_0 was the concentration of template molecules, l was the absorption thickness of cell.

To $\Delta A/b_0^n$ and ΔA drawing can deduce the value of n , thus learned that the action mode of template molecule and functional monomer, that is to say around template molecule has several functional monomer and its effect, so as to reveal the mechanism of molecular imprinting (Figure 3b).

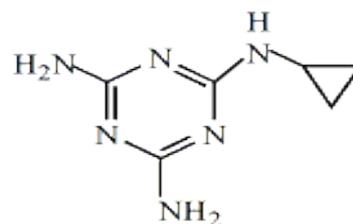
Can be seen from the Figure 3c when $n=2$, $\Delta A/b_0^2$ and ΔA mapping was a straight line, it showed that template and functional monomer within the scope of the concentration the main form for one MEL interact with two MAA form host-guest complexes. The melamine molecular was symmetric structure contains three



Melamine



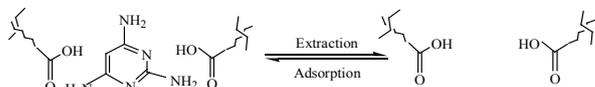
Cyanuric acid



Cyromazine

Figure 2 The structure of MEL, CYA and CYR

functional group (NH_2), but there are only two interact with monomer which could be the influence of the space steric effect. Presumably, melamine molecularly imprinted polymer synthesis route:



FT-IR spectra

The FT-IR spectra of $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (b) and MMIPs (c) were given in **Figure 4**. The characteristic absorption band of Fe-O situated at 467.7 cm^{-1} and 580.6 cm^{-1} in each curves. The strong peak at $1,096.6 \text{ cm}^{-1}$ was attributed to the asymmetry stretching vibration of Si-O-Si bonds. Other absorption bands, such as the Si-O bending vibration peak at 808.6 cm^{-1} and the

Si-O-H vibration peak at around 954.2 cm^{-1} were appeared in the $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ sample (**Figure 4**). Such results confirmed that SiO_2 was indeed coated onto the surface of Fe_3O_4 nanoparticles. The typical peak of $1,730.2 \text{ cm}^{-1}$ represents the stretching vibration of carbonyl groups of the MPS in **Figure 4**. Upon comparing MMIPs to $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$, a bond absorption band at $2,970.3 \text{ cm}^{-1}$ of the MMIPs corresponded to the C-H aromatic stretching vibration of EDMA. Then, the peak of C-H at $1,390.1 \text{ cm}^{-1}$ symmetric bending vibration from methyl groups and C=O bond at $1,731.6 \text{ cm}^{-1}$ stretching vibration increase observably. The appearances of these bands were confirmed that the polymerization was successful.

Thermal stability analysis

Figure 5 depicted the thermogravimetric curves (TGA) of the Fe_3O_4 (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b), $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c), and MMIPs (d).

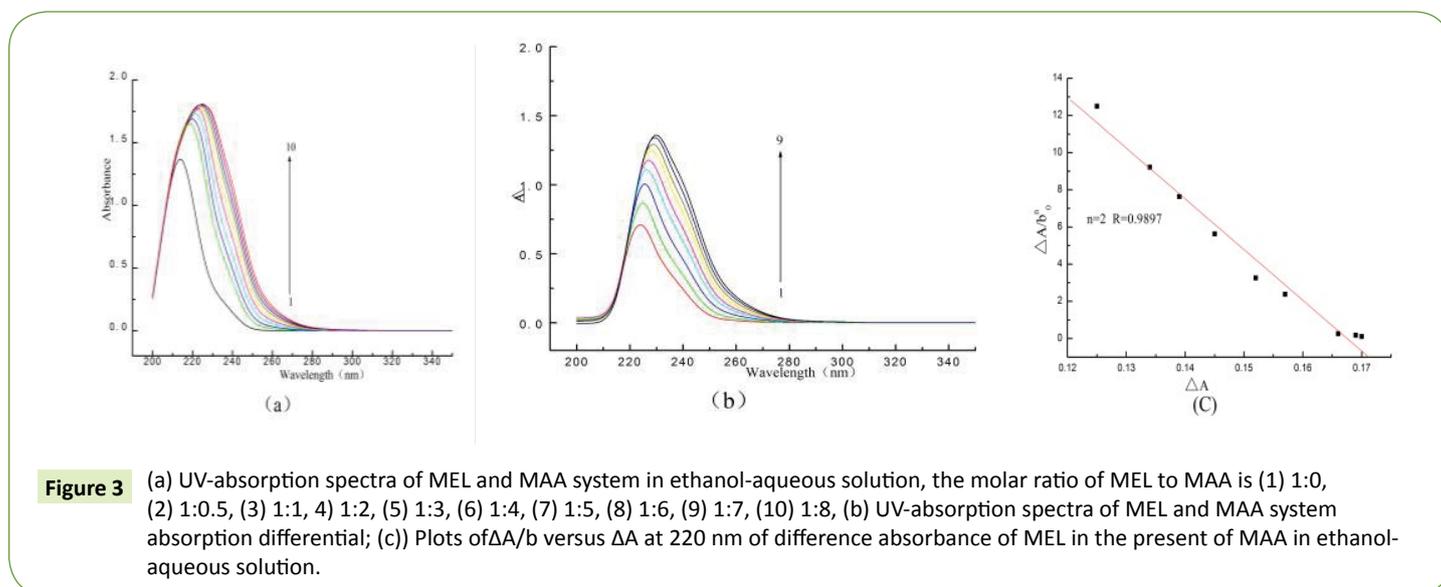


Figure 3 (a) UV-absorption spectra of MEL and MAA system in ethanol-aqueous solution, the molar ratio of MEL to MAA is (1) 1:0, (2) 1:0.5, (3) 1:1, 4) 1:2, (5) 1:3, (6) 1:4, (7) 1:5, (8) 1:6, (9) 1:7, (10) 1:8, (b) UV-absorption spectra of MEL and MAA system absorption differential; (c) Plots of $\Delta A/b$ versus ΔA at 220 nm of difference absorbance of MEL in the present of MAA in ethanol-aqueous solution.

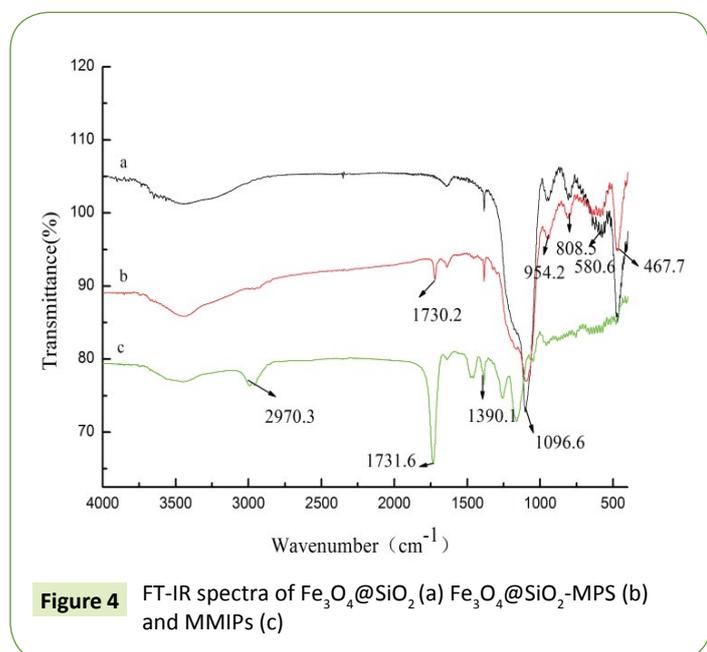


Figure 4 FT-IR spectra of $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (a) $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (b) and MMIPs (c)

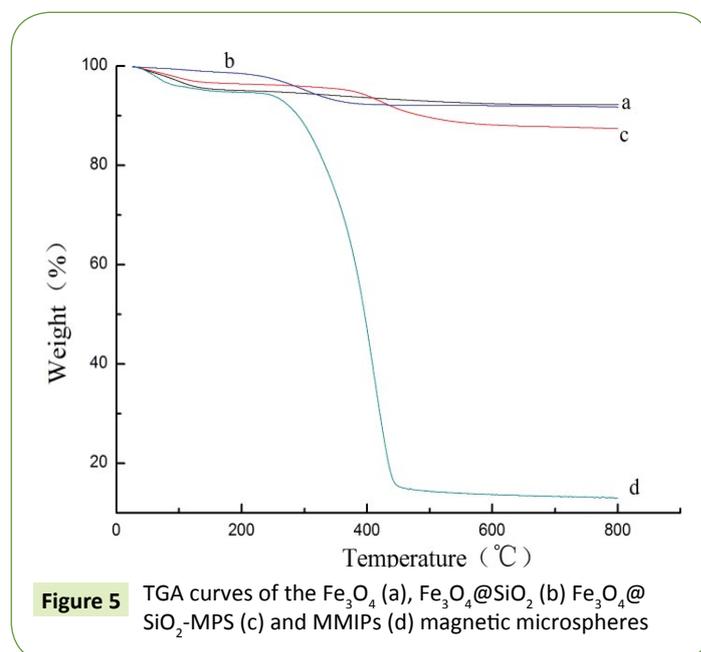


Figure 5 TGA curves of the Fe_3O_4 (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b) $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) and MMIPs (d) magnetic microspheres

As shown in **Figure 5a** illustrated that the Fe_3O_4 (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b), and $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) microspheres have good thermal stability. From room temperature to 800°C , there is only little lost for about 8%. In details, the rate of weight loss for Fe_3O_4 (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b) and $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) owed to remnant ethanol the dehydration in the layer of samples under below 150°C . When the temperature was changed from 400°C to 600°C , the decreased weight might be due to decomposition of organic matter in $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b) and $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c). Especially the weight loss rate of $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) was much higher than the $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b) at nearby 500°C , which demonstrated existence of MPS. From **Figure 5d**, it is obvious that MMIPs had a rapid weight loss rate from 300°C to 500°C , and the decreased weight of MMIPs was approximately 80.4%. The weight loss should be the decomposition of MMIPs.

Magnetic properties

The magnetic properties of the Fe_3O_4 nanoparticles (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b), $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) and MMIPs (d) were characterized by VSM at room temperature and the results are shown in **Figure 6**. The values of saturation magnetization for the Fe_3O_4 (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b), $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) and MMIPs (d) were 60.67 emu/g, 27.10 emu/g, 21.76 emu/g and 3.86 emu/g, respectively. The saturation magnetization value of Fe_3O_4 was similar to that from the literature [22]. And this value was smaller than that of the theoretical specific saturation magnetization of bulk magnetite (92 emu/g [23]). The value of saturation magnetization was decreased due to the small particle surface effect. The saturation magnetization value of the $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ was far less than that of the pure Fe_3O_4 nanoparticles. The decrease in magnetization

value is most likely attributed to the existence of silica shells on the surface of Fe_3O_4 nanoparticles, leading to a weakening of the magnetic moment. The $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ exhibited slightly weak magnetic properties after modified by MPS. The magnetic hysteresis loop of the MMIPs is illustrated in **Figure 6b**. The values of saturation magnetization for the MMIPs were $3.86 \text{ emu}\cdot\text{g}^{-1}$. The saturation magnetization value of the MMIPs is smaller compared with the pure Fe_3O_4 nanoparticles. So the agminate MMIPs can renewably disperse rapidly after the magnetic field is removed. **Figure 6** (inset) shows the photographs of the separation process of MMIPs beads. In the absence of an external magnetic field, a yellow homogeneous dispersion exists. When an external magnetic field was applied, the black particles were attracted to the wall of vial and the dispersion became clear and transparent in a short time. As a result, the MMIPs particles could be easily and quickly separated from a suspension.

X-ray diffraction patterns

The structural properties of Fe_3O_4 nanoparticles (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b), $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) and MMIPs (d) were analyzed by XRD. As shown in **Figure 7**, the discernible six characteristic peaks for Fe_3O_4 ($2\theta=30.2^\circ$, 35.6° , 43.3° , 53.5° , 57.2° , and 62.8°) were observed for all the samples, and the peak positions could be indexed to (220), (311), (400), (422), (511) and (440), respectively, which match well with the database of magnetite in the JCPDS-International Center (JCPDS Card: 19-0629). The peak positions were unchanged during the synthesized process, which indicating that the crystalline structure of the magnetite was essentially maintained. The diffraction peak with $2\theta=22^\circ$ can be assigned to the amorphous SiO_2 for $\text{Fe}_3\text{O}_4@ \text{SiO}_2$, $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ and MMIPs.

Characterized by scanning electron microscopy

The microscopic features of MMIPs was changed before and after adsorption MEL were shown in **Figure 8**. The images of left for fresh before adsorption MMIPs surface was porous, rough and had a large number of mesopores on their surface which were suitable for rebinding or releasing the target molecules from the MMIPs particles. The observations also confirmed that the magnetic polymeric microspheres had been successfully prepared, the right for the saturated adsorption MMIPs, it can be seen that the magnetic material surface is relatively smooth, crisscross lamination reduction, after adsorption MEL surface of magnetic material obvious changed, indicate that the MMIPs has good adsorption (**Figure 8**).

Adsorption capacity

Static adsorption tests were performed on the MMIPs and MNIPs, MMIPs and MNIPs with different initial concentrations of MEL (**Figure 9**). The MMIPs and MNIPs particles were put in the concentrations of MEL solutions ranging from 20 to 120 mg/L, when the concentration of MEL reached 90 mg/L and more, the bound amounts achieved a saturated plateau for MMIPs and MNIPs. The static adsorption capacities of the MMIPs and MNIPs for MEL were 8.74 mg/g and 3.48 mg/g, respectively, which displayed better steric matching with the imprinted molecule MEL, indicating that the MMIPs had a higher affinity than the

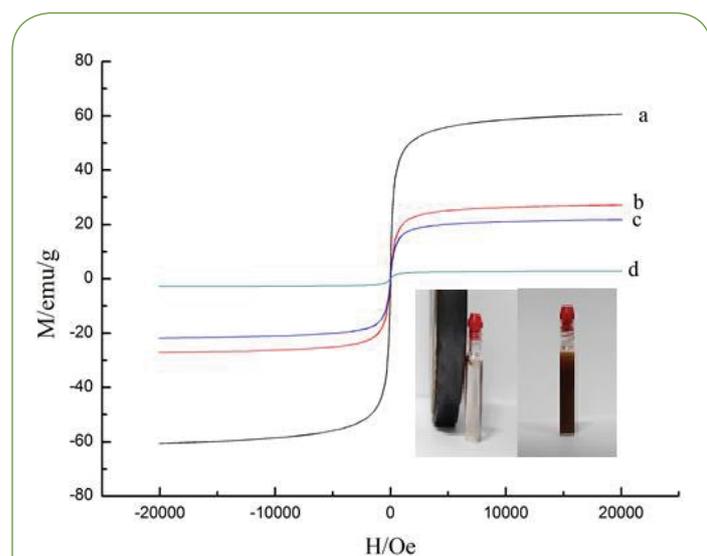


Figure 6 Magnetization curves and a photograph of magnetic molecularly imprinted materials of an external magnetic field
Magnetization curves of Fe_3O_4 (a), $\text{Fe}_3\text{O}_4@ \text{SiO}_2$ (b), $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-MPS}$ (c) and MMIPs (d) magnetic microspheres and a photograph of MMIPs dispersed in the water in the presence (left) and absence (right) of an external magnetic field (c)

MNIPs for the template molecule. The calculated maximum adsorption capacities of the MMIPs exhibited better than the literature value [20]. In general, MMIPs possess both specific and nonspecific binding sites, while MNIPs only has nonspecific binding sites, which enable MMIPs to take up more MEL than MNIPs. The difference in adsorption capacity between MMIPs and MNIPs is equivalent to the contribution from the specific binding sites on MMIPs.

In order to further study the binding properties of the MMIPs beads, Scatchard analysis was performed by using the saturation binding data. As can be seen from **Figure 10**, the Scatchard plot for MMIPs was not a single linear curve, but rather consisted of two linear parts with different slopes, which suggested that there were two different types of binding sites for MMIPs towards linearity MEL molecular. The K_d and Q_{max} values can be calculated

according to the slopes and intercepts of the two linear portions of the Scatchard plot. The results are shown in **Table 1**.

Adsorption kinetics

Figure 11 presents the adsorption kinetics of MEL solution on the MMIPs. Adsorption equilibrium of MMIPs nanoparticles in MEL solution was determined at different adsorption times. The initial concentration of MEL was kept constantly at 90 mg/L. In 10~60 min, the binding amount of MEL increased rapidly and reached an equilibrium after 30 min, the adsorption capacity rapidly increase at the initial stage, and then slow down to reach the adsorption equilibrium.

Selectivity

The adsorption selectivity is an indispensable factor for appreciating the capacities of an adsorbent. To evaluate the selectivity of the MIPs, analogue of cyanuric acid and cyanazine were selected. The initial concentrations of MEL, CYA and CYR used were 90 mg/L. The experimental results were plotted in **Figure 12**, the bound amount of MEL for MMIPs was much higher than that of the other two competitive analogs. Although the structures of cyanuric acid and cyanazine are very similar with template MEL, the MMIPs could still specifically recognize MEL based on the imprinted sites.

The application in milk samples analysis

It was further applied to analyze melamine in milk for demonstrate the applicability of the method. The milk was added by spiking melamine solution at three levels (200.0, 300.0, and 500.0 $\mu\text{g/L}$). The molecularly imprinted materials can be repeatedly used 6 times, the recoveries of MEL in the spiked samples were 85.6% to 104.2%. Detection limits for MEL were within the range of 0.15-3.00 $\mu\text{g/mL}$, which could meet the analysis of trace melamine in milk. The limit of detection (LOD) ($S/N=3$) of this new method was 15 $\mu\text{g/L}$. Simultaneously, the recovery of melamine by the MMIP is high than that of the MNIPs, the resulted demonstrated that the good selection of adsorption of the MMIPs for melamine in the milk (**Figure 13 and Table 2**).

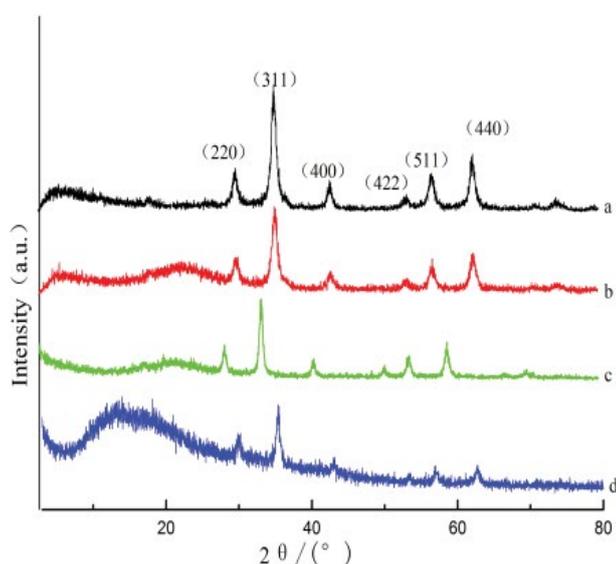


Figure 7 XRD patterns of magnetic Fe_3O_4 nanoparticle (a), $\text{Fe}_3\text{O}_4@SiO_2$ (b), $\text{Fe}_3\text{O}_4@SiO_2$ -MPS (c) and MMIPs (d).

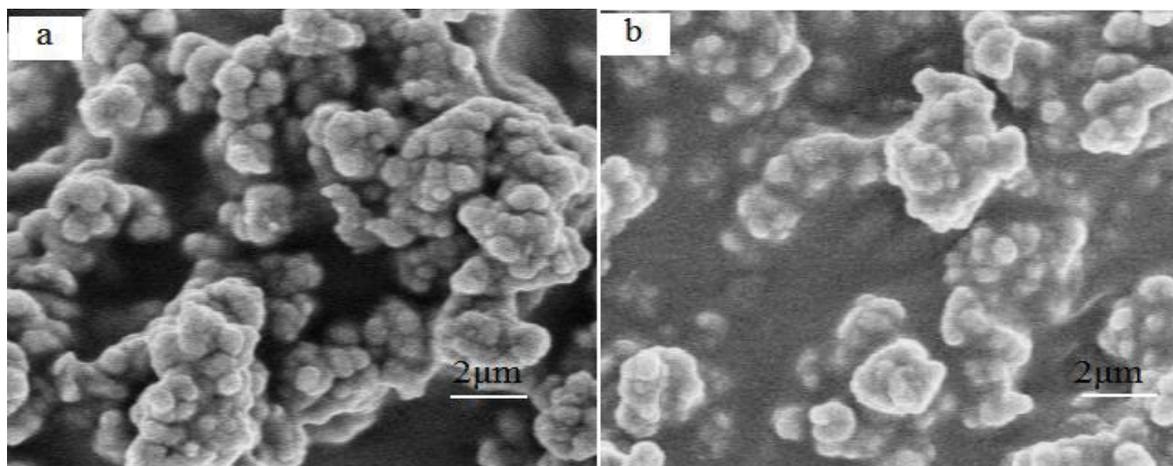


Figure 8 Scanning electron micrographs of before (a) and after (b) MMIPs magnetic microspheres adsorbed MEL

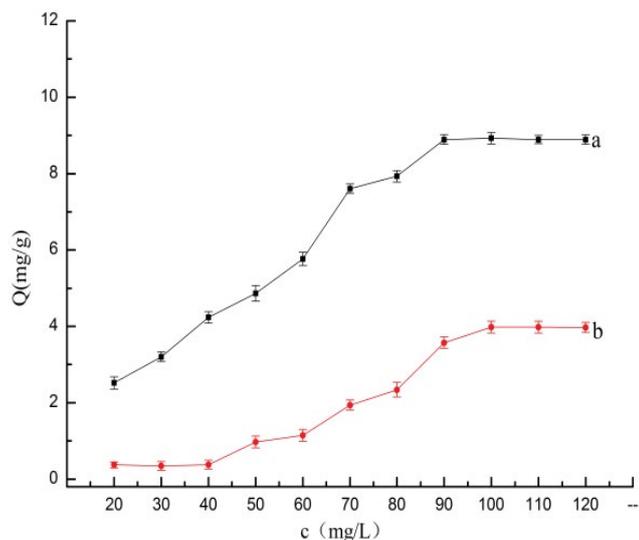


Figure 9 Adsorption capacity curves of MMIPs and MNIPs.

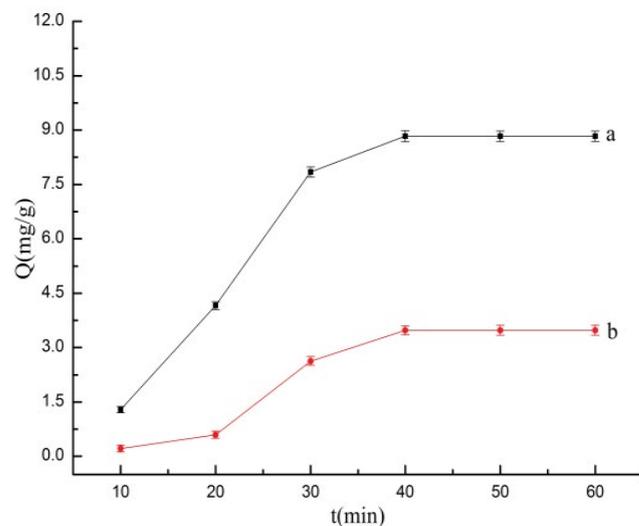


Figure 11 Adsorption kinetics curves of MMIPs and MNIPs.

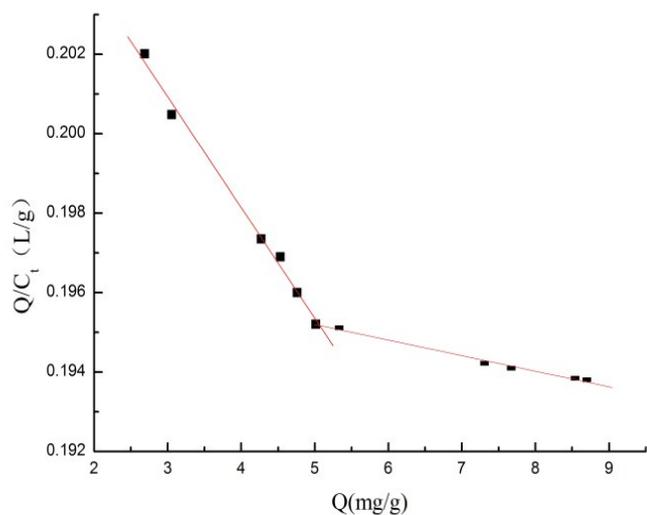


Figure 10 Scatchard analysis of MMIPs

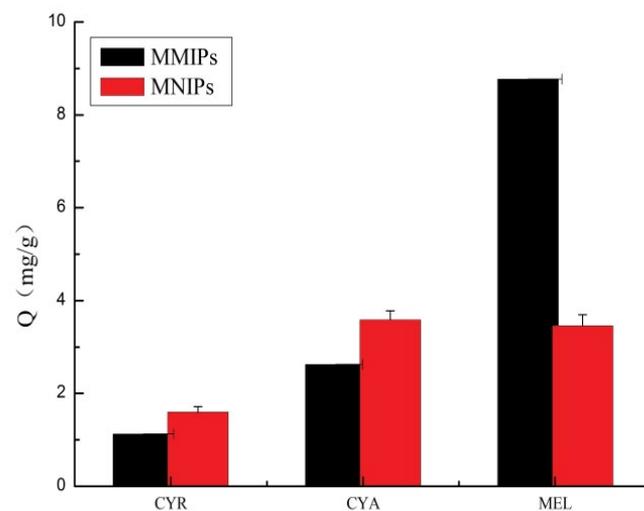


Figure 12 Selective adsorption for MEL, CYA and CYR on MMIPs and MNIPs.

Table 1 The results of the scatchard analysis.

Binding sites	Linearity	K_d (g/L)	Q_{max} (mg/g)
Lower affinity sites	$Q/C_1 = -0.62006 \cdot 10^{-5}x + 0.19883$ ($R^2 = 0.8639$)	161.27	32.07
Higher affinity sites	$Q/C_1 = -0.00272x + 0.20908$ ($R^2 = 0.9236$)	0.3676	0.07687

Conclusions

In the study, the MMIPs using melamine as a template were prepared based on surface modification of the $Fe_3O_4@SiO_2$ nanoparticles by using molecularly imprinting technique. The obtained magnetic microspheres were characterized by FT-IR, TGA, VSM, SEM and XRD. In the rebinding experiment, high

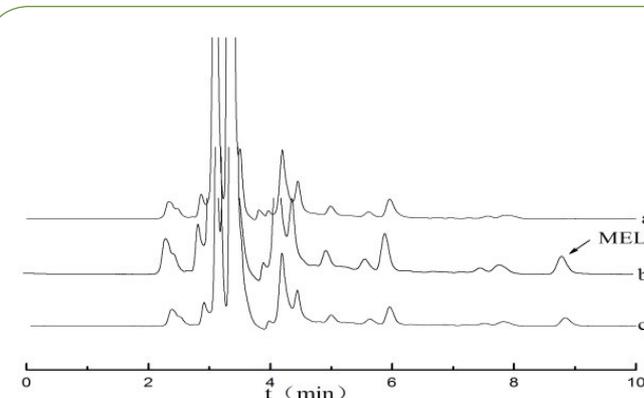


Figure 13 The chromatogram of the milk sample after extraction: the spiked milk sample with 300.0 μ g/L melamine after extraction with MMIPs (b), MNIPs (c), without spiked melamine after extraction with MMIPs (a)

Table 2 Recovery results of spiked melamine in milk (n=3).

Samples	Adding level ($\mu\text{g/L}$)	imprint		nonimprint	
		Recovery (%)	RSD%	Recovery (%)	RSD%
MEL	200	85.6	2.8	72.6	3.7
	300	92.1	3.2	81.7	3.3
	500	104.2	8.3	96.3	9.3

adsorption capacity and good selectivity for melamine were demonstrated by the MMIPs beads. Furthermore, the MMIPs

were successfully applied to selective enrichment coupled with HPLC analysis for melamine in spiked samples. In conclusion, the MMIPs not only provided a convenient separation with the magnetic nanoparticles, but it also overcame problems of low selectivity with MMIPs. So a novel method combining MMIPs with HPLC analysis was established to determine the melamine.

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