Analytical Methods

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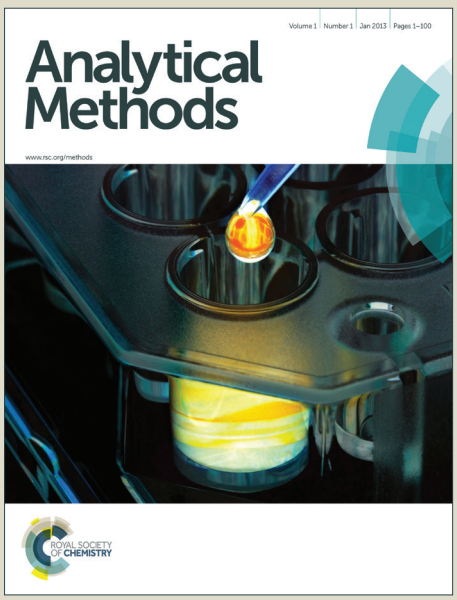

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Synthesis and characterization of molecularly imprinted polymers with modified rosin as cross-linker and selective SPE-HPLC detection of basic orange II in foods

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Abstract: Basic orange Ⅱ (BOⅡ) is one of alkaline azo dyes, which is listed as the prohibited substance in food additives. In order to develop a novel molecularly imprinted polymer (MIP) with modified rosin as cross-linker for selective solid-phase extraction (SPE) of BOⅡ in foods, MIP was synthesized using BOⅡ as the template molecule, acrylamide as the functional monomer, and maleic rosin glycol acrylate（MRGA） as the cross-linking agent. The MIP was characterized using a scanning electron microscope, Fourier transform infrared spectrometer and thermogravimetric analysis. In comparison to the imprinted polymers prepared by the traditional cross-linker, the MIP showed a highly imprinting capacity, significant selectivity, hardness and toughness; could be used as a SPE material and detect illegal addition BOⅡ in food. In the sample with spiked level of 5-11 mg/kg, BOⅡ in foods revealed an average recovery rate of 68.43-80.25% with precision (relative standard deviation) less than 1.2%.

Keywords: Basic orange Ⅱ, molecularly imprinted polymer, solid-phase extraction, HPLC, modified rosin as cross-linker

Introduction

Basic orange Ⅱ (BOⅡ), 2,4-azodianiline, is one of alkaline azo dyes, which are mainly used for leather, textile and wood industries. The ingestion, inhalation or skin contact of BO Ⅱ can result in acute and chronic poisoning. According to relevant animal data, BOⅡ has high carcinogenicity and teratogenicity.[1,2] Under the "Health Standards of Food Additives in China" and "Chinese Food Safety Law", BOⅡ is listed as the prohibited substance in food additives.[3,4] Compared with other water-soluble dyes such as tartrazine and sunset yellow, BO Ⅱ reveals easier staining in foods and more difficult fading. However, in order to make more profits by some unscrupulous traders, BOⅡ is used in soybean products, fish and chili power, which can result in a serious threat to the health of consumers.[5,6] Currently, the detection of BOⅡ in fish, chili powder, soybean products and seasonings and other foods has been reported.[7-10] The domestic standard method for the determination of BOⅡ was GB/T 23496-2009. "Determination of forbidden materials in foods-Dyes of basic orange-High performance liquid chromatography methord"[11] These detection methods include initial liquid-liquid extraction (LLE) or solid phase extraction (SPE) for sample purification or enrichment and detection by high performance liquid chromatography (HPLC) or mass spectrometry. SPE is characteristics of simple operation, fast separation, low cost and easy automation, which can be used in HPLC and mass spectrometry. But the selectivity of commercial SPE sorbents such as bonded silica, organic polymer, inorganic matrix and graphene to the analytes during SPE is not ideal. Therefore, the development of adsorbents with high selectivity and high adsorption capacity for SPE is highly desired.

Molecular imprinting technology (MIT) is the most promising technology for the purification and enrichment of samples. Molecularly imprinted polymer (MIP) with high selectivity and affinity to target molecules in the field of analytical chemistry has attracted considerable attention. However, the commonly used cross-linking agents such as ethylene glycol dimethacrylate (EGDMA) are limited by their molecular structures. During synthesis, the instability of mechanical properties, low cross-linking degree, and large required amount of cross-linking agents are present[12] so that synthesized MIP has strong diffusion-controlled behavior.[13] Because of the cross-linking agent modified by rosin has phenanthrene skeleton[14],

the synthesized polymer has stable performance, less consumption and high cross-linking degree. Therefore, the MIP made from ethylene glycol maleic rosin ester as cross-linking agent has the characteristics of moderate hardness, appropriate toughness, high separation efficiency and good stability.[15-17]

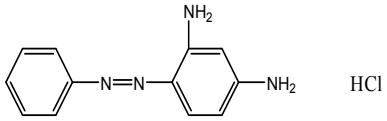
In this study, we chose BOⅡ as a template molecule, acrylamide as a functional monomer, maleic rosin glycol acrylate （MRGA） as a cross-linking agent to synthesize MIP with phenanthrene skeleton through suspension polymerization method. Meanwhile, the morphology and structure of MIP were explored using a scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FT-IR). In addition, the adsorption capacity of MIP was investigated by combined balance experiments and Scatchard analysis. Moreover, the selectivity of MIP was evaluated by the re-combination capability between MIP and BO Ⅱ or other azo dyes. Due to the specific adsorption capability of MIP to BOⅡ, MIP can be used for selective solid-phase extraction, enrichment and detection of BOⅡ, which will provide a reference method for the inspection of food safety.

Experimental

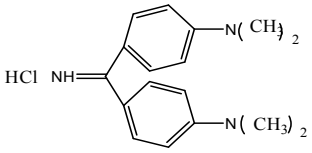
Materials and reagents

Basic orangeⅡ, Auramine O, Rhodamine B, trimethylolpropane trimethacrylate(TRIM), N,N-methylene-bis-acrylamide(MBA) were all purchased from Shanghai Jingchun Reagent Co., Ltd. (China); ethylene glycol dimethacrylate (EGDMA) was obtained from Fushun Anxin Chemical Co., Ltd.(China); sodium dodecyl sulfate (SDS) was obtained from Tianjin Dibo Chemical Company (China); azobisisobutyronitrile (AIBN) was purchased from Shanghai Sihewei Chemical Co., Ltd.(China); acrylamide (AM), methyl methacrylate (MMA), methacrylic acid (MAA), ethyl acetate, n-hexane, methanol, ethanol and acetic acid were all purchased from Shantou Xilong Chemical Company(China); Acetonitrile was obtained from Fisher Scientific(USA). Highly purified water was obtained from a Pro Water System (American Millipore Company). All reagents used were of analytical or HPLC grade and used without further treatment. maleic rosin glycol acrylate (MRGA) was made by our research group[14]. Soybean oil was purchased from Guangxi Molaoye Foods Co., Ltd (China). Food samples were randomly purchased from some local supermarkets (Nanning ,

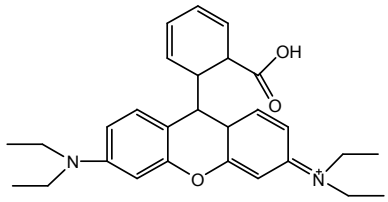
China).



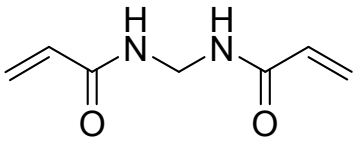
Basic Orange Ⅱ (BOⅡ)



Auramine O

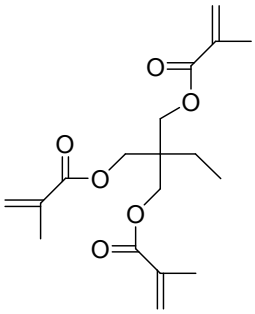


Rhodamine B



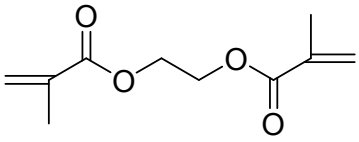
N,N-methylene-bis-acrylamide

（MBA）



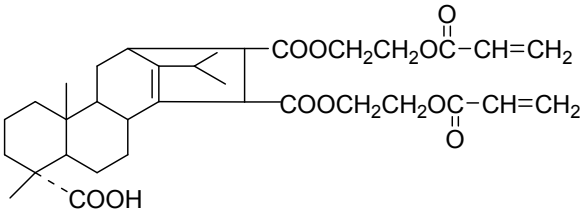
trimethylolpropane trimethacrylate

（TRIM）



ethylene glycol dimethacrylate

（EGDMA）



maleic rosin glycol acrylate

（MRGA）

Fig.1. Chemical structures of the molecules in the study

Instrumentation and conditions

Morphology of MIP and non-imprinted polymer (NIP) was examined by S-3400N scanning electron microscopy (Hitachi, Japan). IR spectra (4000-400 cm^{-1}) of MIP and NIP were recorded by Magna IR550-type Fourier infrared spectrometer (Nicolet, USA). Thermal gravimetric analysis was conducted by TG209F1 thermal gravimetric analyzer (Germany NETZSCH Company). SPE was performed in 12-Ports AHO-6023-type solid-phase extraction system coupled with vacuum-controlled valve and Teflon adapter (US Phenomenex Company). UV-visible absorption spectra were measured by UV-VIS916 UV-visible spectrophotometer (Australia GBC Company).

HPLC analysis was conducted on Shimadzu LC-20A HPLC system coupled with an SPD-20A UV-Vis detector, LC-20A pump, HT-340K oven and LC solution 15C chromatography workstation. The separation of the analytes was conducted on Phenomenex Gemini C18 column (5 μm particle size, 250 mm × 4.6 mm) from US Phenomenex Company. The detection conditions included acetonitrile-water (60/40, V/V) as the mobile phase with flow rate of 1.0 mL/min, detection wavelength of 449 nm, column temperature of 40 °C, and injection volume of 20 μL.

Preparation of BOⅡ **MIP**

0.0497 g (0.2 mmol) BOⅡ was weighed in a small beaker and dissolved in appropriate amount of ethanol. Then, 0.2843 g (4 mmol) acrylamide, 3.684 g (6 mmol) maleic rosin glycol acrylate, 0.25 g azobisisobutyronitrile, 2 mL soybean oil and 10 mL ethyl acetate were sequentially added to the dissolved BOⅡ solution. After ultrasonic treatment for 20 min, the mixture was poured into 100 mL 0.10% aqueous solution of SDS in a three-neck flask under stirring (210-230 rpm) at room temperature. Then, the reaction system was gradually heated to 75 °C for 1 h. After connecting distiller, the reaction system was slowly heated to 85 °C to complete the fractionation of the organic solvent. Finally, the reaction system was slowly heated to 95 °C with continuous heating for 1 h. Subjected to filtration, raw polymer particles were isolated. The polymer particles were then placed in the filter cartridge through gradient elution by acetic acid-ethanol at the volume ratios of 80/20, 50/50, 20/80 and 0/100, respectively. After removing BOⅡ through Soxhlet extraction, the final

residual solvent was washed with distilled water at room temperature. After drying at 60 °C, the MIP was obtained. NIP was synthesized without template molecules using similar steps. The average particle diameter of the polymers was 180-250 μm.

Isotherm adsorption experiment

0.2 g of MIP or NIP was weighed in 100 mL conical flask with a stopper, respectively. After adding 20 mL of BO□ at various concentrations of 0.1- 4.0 mg/mL. The mixture was placed on a shaker with shaking at 80 rpm at room temperature（30 °C）for a certain time. Until the polymer reached to saturation, 0.4 mL of saturated solution was accurately transferred by pipette to 100 mL volumetric flask. Setting up to the volume with distilled water, the absorbance was determined by UV-visible spectrophotometer at 449 nm. Equilibrium binding capacity was calculated by following equation:

$$Q = V(C_0-C_e)/W \qquad (1)$$

Where: Q is the adsorption capacity (mg/g); C_0 and C_e are initial concentration and equilibrium concentration (mg/mL) of BO Ⅱ in ethanol-water(45:55,v/v) solution, respectively; V is the solution volume (mL), and W is polymer mass (g).

Adsorption kinetics experiment

0.5g of MIP or NIP was weighed in 250 mL conical flask with a stopper, respectively. Then, 100 mL of 2 mg/mL BO Ⅱ solution was added to conical flask on an oscillator with 80 rpm at room temperature（30 °C）. In the period of 10-300 min, the absorbed solution was transferred in a certain time interval by pipette and brought to the designed volume using distilled water. The binding capacity of MIP and NIP to BO Ⅱ was determined by UV-visible spectrophotometry.

Identification of MIP and NIP characteristics

0.2 g of MIP or NIP (40-60 mesh) was weighed in 100 mL conical flask with a stopper, respectively, and then added 20 mL of BO Ⅱ, Rhodamine B and Auramine O or mixture solution with BO Ⅱ and Auramine O (or Rhodamine B), respectively. According to adsorption experiments in *Section 2.4.1*, 0.4 mL of supernatant was transferred to 100 mL volumetric flask and set up the volume using distilled water. The prepared solution was filtered with 0.45

μm filter membrane for HPLC. The competitive adsorption capacity of MIP and NIP to BOII was detected by HPLC. Imprinting factor (α) [18, 19] and separation factor (β) [20] were calculated as follows:

$\alpha = Q_{MIP}/Q_{NIP}$ (2)

Where: α is imprinting factor; Q_{MIP} is the binding capacity of MIP on template molecule; Q_{NIP} is the binding capacity of NIP on template molecule.

$\beta = Q_{template\ molecule}/Q_{competitive\ molecule}$ (3)

Where: β is separation factor; $Q_{template\ molecule}$ is the adsorption capacity of MIP on template molecule; $Q_{competitive\ molecule}$ is the adsorption capacity of MIP on competitive molecule.

SPE of MIP

0.30 g of MIP particles was filled in 6 mL empty SPE tube with filling height of approximately 1 cm. The top and bottom of filler have polyethylene frits. The ethanol as the homogenate was used for polyethylene SPE column with tight sieves. Sequentially adding 3-5 mL of methanol solvent, 3-5 mL of ethanol-water (45:55, V/V).2 mL of BO II standard solution (or the spiked solution) in ethanol-water (45:55, V/V) was passed through the cartridges at a flow rate of 0.2 mL/min. Then, 3 mL of ethanol-water (20:80, V/V) was used to wash the column. Anhydrous ethanol was used to elute (2 mL×3 times). The collected elution was filtered using 0.45 μm filter membrane for HPLC analysis.

Purification and detection of BO II in food samples

Food samples were purchased from a local supermarket. 2.0g food sample was weighed in 100 mL conical flask, and dissolved in 10 mL of n-hexane. After ultrasonic treatment for 1-2 min, the hexane layer was discarded. After drying, 20 mL of ethanol was added and subjected to ultrasonic extraction for 20 min. After standing for 2-3 min, 10 mL of supernatant in 100 mL eggplant-shaped flask was evaporated to dry under reduced pressure at 45 °C.[11] 10 mL of ethanol-water (45:55, V/V) was subjected to ultrasonic treatment for 1-2 min and centrifugation at 10000 r/min for10 min. Then, 2 mL of supernatant passed through SPE column slowly. The separation and detection of BOⅡ in foods was conducted using

similar SPE method of MIP. The spiked level of the sample was 5-11 mg/kg.

Results and discussion

Optimization of synthesis conditions

MIP preparation process includes self-assembly, polymerization and template removal.[21] Self-assembly is the formation of template-monomer complex between BO II and AM in the solvent through non-covalent interaction. Polymerization is the formation of polymer through thermal polymerization reaction during the addition of cross-linking agent and initiator. Template removal is the elution process of BOⅡ at the recognition sites through a mild chemical method. After eluting BOⅡ, three-dimensional holes with same size and shape as BOⅡ were left in the polymer, and also contained functional groups provided by complementary monomers of BOⅡ.

The imprinting efficiency and selectivity of MIP on BOⅡ depend on a variety of factors. The preparation process of MIP was optimized by changing corresponding factors such as functional monomers, cross-linking agent proportion, porogen, solvent type, initiator amount, reaction temperature, reaction time, stirring speed, dispersant amount and elution condition. The preparation and identification mechanism of BOⅡ MIP are shown in Figure 2.

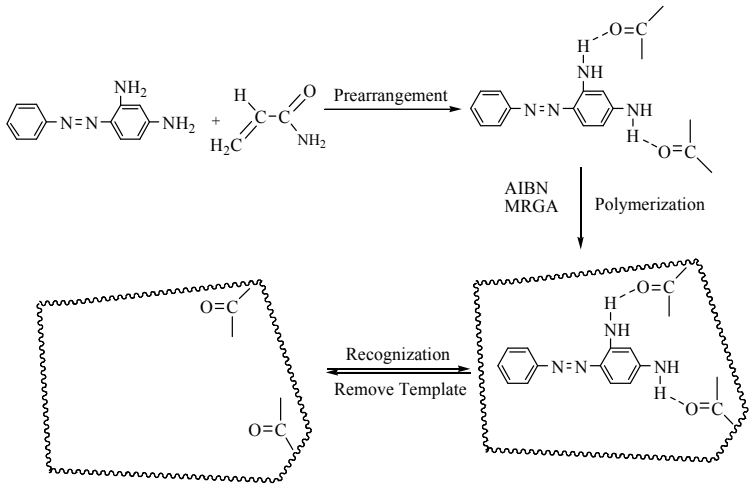


Fig.2. Schematic illustration of the preparation and molecular recognition of Basic Orange Ⅱ imprinted polymer

When BOⅡ and AM were mixed at the ratios of 0.1:0.5 or 0.1:4, MIP revealed a lower adsorption capacity and imprinting effect on BOⅡ. While BOⅡ and AM at the ratios of 0.1:1 or 0.1:3, MIP exhibited an enhanced adsorption capacity on BOⅡ although the imprinting efficiency remained a low level. In contrast, while BOⅡ and AM at the ratio of 0.1:2, MIP had both higher adsorption capacity and better identification capability on BOⅡ.

The change in cross-linking agent ratio can obviously improve the hardness and thermal stability of MIP. When AM and MRGA were at a molar ratio of 2:3, MIP had higher adsorption capacity (28.68 mg/g), stronger thermal stability (softening point 276 °C) and better imprinting effect (imprinting factor α = 2.6) on BOⅡ. In the following study, the optimal ratio among BOⅡ, AM and MRGA was 0.1:2:3 for the preparation of BOⅡ MIP complex.

In the present study, ethyl acetate was chosen as the organic phase solvent, and soybean oil was used as the porogen, which can fully dissolve the polymerization monomer and the initiator in the organic phase, and can result in the pore formation in polymers during the polymerization reaction, thus contributing to the adsorption of MIP on the surface and inside on template molecules.

During the suspension polymerization process, the stirring rate and the dispersant amount revealed important influence on the morphology and size distribution of polymer particles. The reactive monomer was dispersed into small droplets by stirring, which promoted the flow of materials, enhanced the uniformity of heat transfer, accelerated the complete polymerization and maturation of polymers and prevented the sedimentation and adhesive of polymer particles. Dispersants reduced the surface tension of reactive monomers and improved the droplet formation in the presence of stirring, thus protecting the dispersed particles and avoiding the aggregation of dispersed particles. Through experimental screening, the optimal conditions including 0.10% SDS and stirring speed at 230 rpm could result in uniform size for MIP particles.

During suspension polymerization process, particle size and morphology of polymers are associated with polymerization temperature**.** In the present study, AIBN, as a radical initiator, has decomposition temperature of approximately 60 °C. According to the properties of AIBN, temperature-programmed synthesis mode was selected. At room temperature, the initial

stirring for 20 min for the reaction system can result in the dispersion of polymers into small droplets. The optimal processes including slow warming to 75 ℃, constant temperature heating for 1 h, continuous warming to 85 ℃, complete evaporation of organic solvent in reaction system, and slow warming to 95 ℃ for 1 h heating, can provide the uniformity size of synthesized polymer particles. After the polymer curing, the reaction was terminated. Under these optimal conditions, unformed polymer particle size, moderate hardness and appropriate tenacity, as well as excellent heat resistance were achieved.

Finally, acetic acid-ethanol solution was used as the eluent for gradient elution of MIP to remove template molecules in the polymer matrix and MIP was obtained. Using same preparation processes, NIP was achieved in the absence of BOⅡ. The average particle diameter of the polymer was 180-250 μm.

Currently, in the field of molecular imprinting technique, few functional monomers and cross-linking agent types limit the development of molecular imprinting technique. Therefore, the development and utilization of novel functional monomers and cross-linking agents are highly desired. Rosin has phenanthrene skeleton and large molecular structure. Its modified product as a cross-linking agent can improve the hardness, toughness and thermal stability of MIP. Meanwhile, its natural properties and non-toxicity reveal good application prospects.[22, 23] In the present study, on the basis of modified rosin (MRGA, relative molecular weight of 614) as a cross-linking agent for the synthesis of MIP containing BOII, the BOII MIP was synthesized through trimethylolpropane trimethacrylate(TRIM, relative molecular weight of 338), ethylene glycol dimethacrylate (EGDMA, relative molecular weight of 198), N, N-methylene-bis-acrylamide (MBA, relative molecular weight of 154) and other cross-linking agents. The physicochemical properties of these polymers were compared to confirm the advantages of rosin-modified cross-linking agent. The structures of 4 kinds of cross-linking agents were shown in Figure 1.

Under the conditions with same ratio ([template]/[functional monomer]/[cross-linking agent] = 0.2:4:6) and synthetic conditions, the synthesized polymers using TRIM, EGDMA or MBA as the cross-linking agent cannot form good particles, but can produce jelly-like or powdery polymers with low cross-linking degree and hardness. The main reason is that three common cross-linking agents are straight-chain molecules, and their molecular weights are

small. They can execute obvious support function for polymer skeleton at the condition of low application amount of cross-linking agents; in contrast, rosin-modified cross-linking agent can complete this supporting function. According to literature reports, during the synthesis of MIP, the low amount of cross-linking agents can result in low cross-linking degree of MIP and the dissolution of polymers in organic solvents.[24] In order to improve the hardness, cross-linking degree and durability of polymers, the amount of cross-linking agents should be greatly increased, which is the real reason that [cross-linking agent]/[functional monomer] at the ratio of 20:4 has been reported in many literatures.

Due to the ring structure of phenanthrene and large molecular weight of rosin-modified cross-linking agent, a small amount of rosin-modified cross-linking agent can achieve an ideal skeleton-supporting effect. Therefore, the synthesized polymers using this cross-linking agent are characteristics of high hardness, excellent toughness and good resistance to organic solvents. The rosin-modified cross-linking agent is a novel and green member in the cross-linking family for molecular imprinting technique.

Morphology and structure of MIP

The structure of MIP and NIP were investigated by FT-IR spectroscopy (Figure 3). The FI-IR absorption spectra of MIP and NIP have almost identical characteristic peaks, indicating that the template has been removed completely. Due to the polymerization of AM and MRGA, the dimers of AM and the doublet peak of AM for $-NH_2$ disappeared. In MIP and NIP, the wide peaks in the range of 3200-3600 cm^{-1} should be the absorption peaks from the stretching vibration of OH and NH in AM-MRGA polymers. The absorption peaks of C=O in AM and MRGA were at 1731 cm^{-1}. The absorption peaks of C-H in methyl groups of MRGA were at 2951 cm^{-1} and 2869 cm^{-1}. All of these results revealed the polymerization reaction between functional monomers of AM and cross-linking agent MRGA to generate polymers such as MIP and NIP.

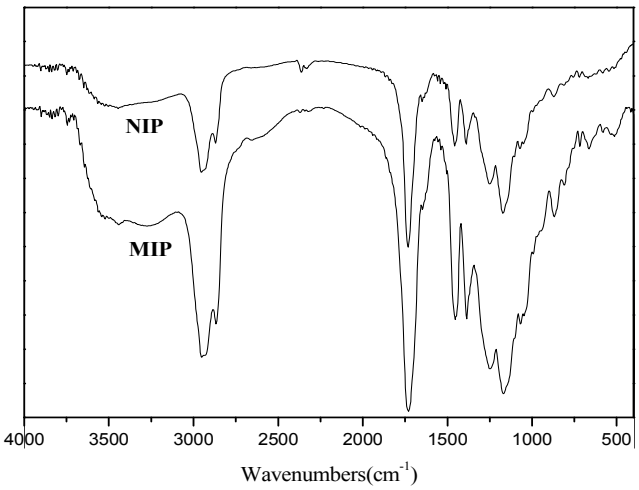


Fig.3. FT-IR spectra of MIP and NIP

The size and morphology of MIP was investigated by scanning electron microscope (Figure 4). As shown in Figure 4(A), MIPs are uniform spherical particles with an average diameter of approximately 200 μm. Meanwhile, the sphere surface was covered with dense holes (Figure 4(B), which provided inner channels for target molecules. When cutting the microsphere surface, the inner sphere revealed honeycomb shape (Figure 4(C). Its unique morphology on the surface and inner part of sphere provided more holes for the distribution of imprinting molecules, correspondingly improving the specific adsorption performance of imprinting polymers.

Fig.4. SEM images of MIP at (A) ×40, (B)×270, and (C)×3000 showing the presence of pores

Thermodynamic stability of the polymer was investigated also, and the results are shown

in Figure 5. As shown in Figure 5, the TG curve indicated that the mass of polymers revealed a decrease by 0.49% at 91.0 °C, which may be due to the residual moisture in polymers. At 279 °C, the polymers exhibited an obvious weight loss so that this temperature is the initial decomposition temperature. The half-life temperature of the polymers is 413.3 °C. In addition, the end temperature was 511.0 °C. On the other hand, based on the curve of DTG, the temperature point (T_p) for the maximum weight loss was 430.0 °C, which revealed the weight loss rate of 69.61%. In the range of 260-480 °C, MIP had two processes of weight loss, which was due to the production of co-polymers between AM and MRGA. The results showed that the prepared MIP had good thermal stability, which was in accordance with the temperature for the application of packaging materials during solid phase extraction.

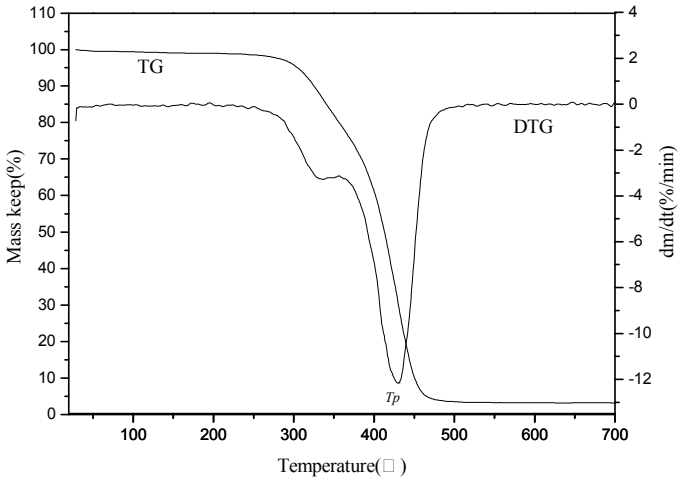


Fig.5.The TG-DTG curves of MIP at a heating rate of 10□.min^{-1} from room temperature to 700□ under N_2 atmosphere.

Selection of solvents for adsorption experiments

The effect of solvent type on MIP performance is focus on the action between MIP and functional groups of polymers. Solvents can result in the inactivation of functional groups and occupancy of the holes in imprinting molecules, thus interrupting the binding between template molecules and functional groups. In addition, solvent molecules can influence their hydrogen bond formation with template molecules and reduce the interaction between

template molecules and functional groups. Finally, solvent molecules can reduce the adsorption performance of MIP to template molecules.

In order to screen a suitable solvent to realize the re-combination between MIP and template molecules, several solvents are subjected to trial exploration. According to the solubility of BOⅡ, ethanol-water solution was the optional. As shown in Figure 6, 45% ethanol can result in the maximum adsorption capacity of MIP on template molecules and obvious imprinting efficiency. Therefore, 45% ethanol was chosen as the absorption solvent in the following experiments.

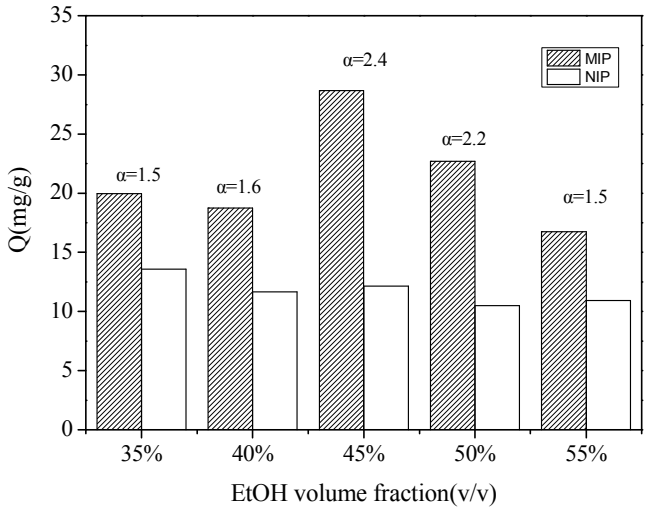


Fig.6. Effect of ethanol volume fraction on the adsorption amount. C_0=2.0mg/mL

Adsorption kinetics

In adsorption kinetics experiments, static combination method and Scatchard analysis were used to explore the molecular recognition properties of MIP on BOⅡ. Figure 7 shows the adsorption kinetic curves of MIP and NIP at the concentration of 2.0 mg/mL on BOⅡ.

As shown in Figure 7, the adsorption capacity revealed an increase as the extension of time. Moreover, MIP had the faster adsorption rate. During the initial 60 min, the adsorption amount revealed a quick increase and the adsorption reached the equilibrium at the time point of 180 min. At the adsorption equilibrium status, the adsorption capacity of MIP and NIP on template molecules was 30.74 mg/g and 13.74 mg/g, respectively. Although MIP and NIP had

same compositions, their spatial structures had an obvious difference. MIP structure contains complementary functional groups and holes with BOⅡ; meanwhile, it has "molecular memory" function on template molecules. Therefore, MIP has strong affinity to template molecules, but NIP does not have this property.

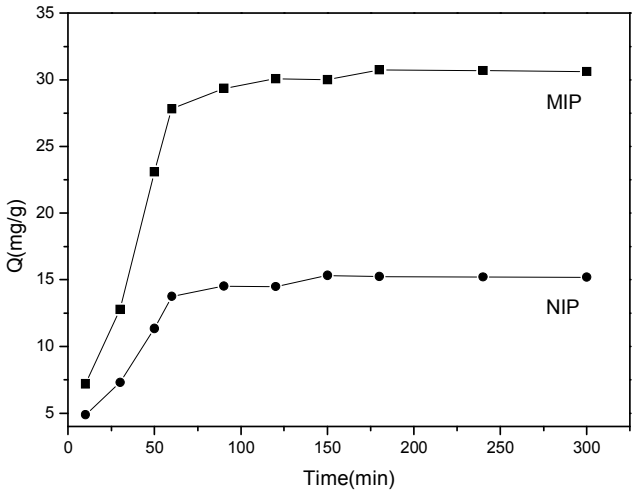


Fig.7. The curves of adsorption kinetics for Basic OrangeⅡ

Adsorption thermodynamics

In the concentration range of 0.10-4.00 mg/mL (initial concentration), isotherm line of static binding of MIP to BOⅡ was determined, as shown in Figure 8. The results showed that the adsorption capacity revealed an increase as the increase of initial concentration.

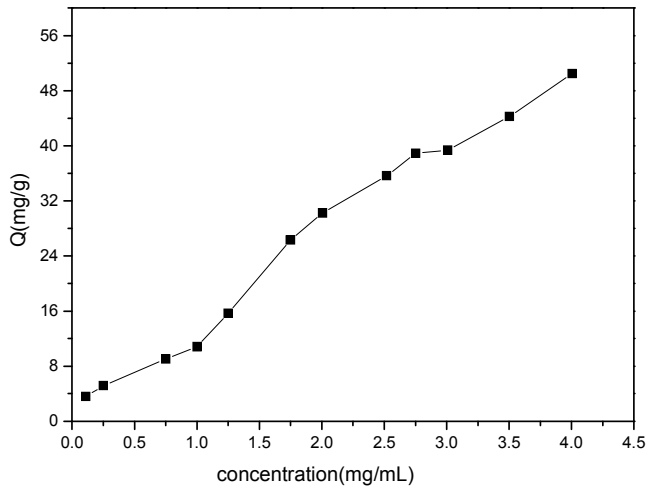


Fig.8. Binding isotherm of the MIP for Basic Orange Ⅱ

The Scatchard binding isotherm equation was used for further data processing, and MIP adsorption characteristics were evaluated. Scatchard equation was as follows:

$$Q/C_e = (Q_{max}-Q)/K_d. \quad (4)$$

In the above equation, Q is equilibrium adsorption amount (mg/g) of MIP to BOⅡ; C_e is free analyte concentration (mg/mL) of BOⅡ at the equilibrium status; K_d is the dissociation constant of the binding point. Scatchard model analysis results were shown in Figure 9. In the Scatchard plot, Q/C_e revealed a non-linear relationship with Q, but two parts of the diagram exhibited a good linear relationship, suggesting that MIP revealed two different binding sites to BOⅡ including low-affinity site and high-affinity site. The possible reason is the different force between functional monomer and template molecule during the preparation of imprinted polymer, thus forming different structures in imprinted hole. Meanwhile, there is also a certain degree of surface adsorption. In Figure 9, two linear equations were established: $Q/C_e = -4.24Q + 58.58$ ($R^2 = 0.9950$); $Q/C_e = -0.19Q + 22.60$ ($R^2 = 0.9414$). By using two linear equations obtained by the slope and intercept, the high affinity binding sites for K_{d1} = 0.24 (mg/mL), Q_{max1} = 13.82 mg/g; low-affinity binding sites for K_{d2} = 5.23 (mg/mL), Q_{max2} = 118.17 mg/g.

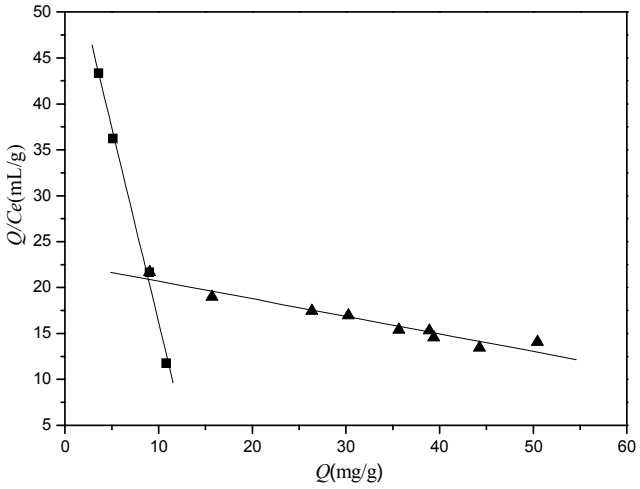


Fig.9. Scatchard analysis of the binding of Basic Orange Ⅱ onto MIP

Evaluation for specific adsorption of MIP

In order to confirm specific imprinting effect of MIP on BOⅡ, two azo molecules such as Rhodamine B and Auramine O in Figure 1 were used as the structural analogues. The adsorption results of MIP and NIP with 20 mL of 2.0 mg/mL BOⅡ or its analogues with similar structures were shown in Figure 10. The adsorption characteristics of MIP on BOⅡ were evaluated by imprinting factor (α).

Adsorption amount is the indicator for the adsorption capability of the polymer. As shown in Figure 10, MIP revealed significantly higher adsorption amount on BOⅡ than Rhodamine B and Auramine O ; however, NIP did not reveal the difference in adsorption amount on three substances. On the basis of imprinting factor α, the imprinting factor α of MIP on BOⅡ was obviously larger than Rhodamine B and Auramine O, suggesting that MIP had good recognition and adsorption properties to BOⅡ, but NIP had surface adsorption to three substances and no characteristic recognition.

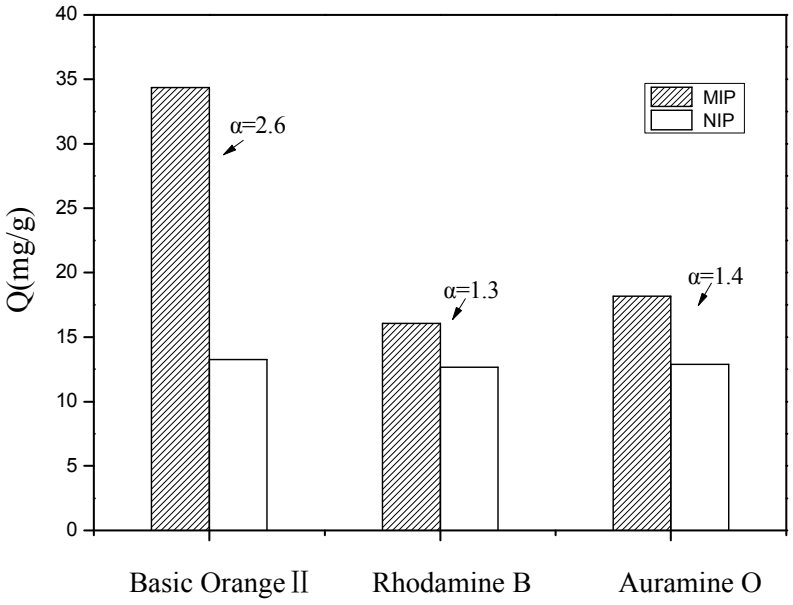


Fig.10. Adsorption capacity of MIP and NIP in single (Basic Orange II, Rhodamine B and Auramine O) azo dye with the initial concentration of 2.0mg/mL for each compound.

Selectivity of MIP separation

In order to confirm the selectivity of MIP for BOⅡ, two azo molecules such as Rhodamine B and Auramine O in Figure 1 were used as the competitive molecules. Both azo substances were mixed with BOⅡ respectively, to prepare the mixture solution with concentration of 2.0 mg/mL for each compound . The adsorption of MIP on three substances was evaluated, as shown in Figure 11. The selective separation of MIP in mixture solution was evaluated by separation factor (β).

Figure 11 shows, in A and B mixture solution, the binding capacity of MIP to BOⅡ was much higher than another competitive binding molecule, which revealed a larger β value. Meanwhile, the α value of BOⅡ was significantly higher than that of competitive binding molecule, suggesting that MIP revealed relatively higher affinity than its analogues. Due to the difference in molecular structures and functional groups as well as steric effect, BOⅡ is suitable for fitting to the cavities of imprinted polymers. In addition, Figure 11 revealed that

the binding amount of MIP and NIP in the mixture solution on template molecule (BOⅡ) was lower than a single solution, which is due to the interaction binding sites in MIP and NIP and competitive molecules in mixture solution. However, the shape, size and spatial distribution of binding sites in imprinted polymers are complementary to template molecules, so the template molecules have the dominant to occupy the binding site when compared with other azo dye molecules. NIP has no specific adsorption on three kinds of azo dyes so that each azo dye has the same chance to occupy the binding site. These results suggested that the adsorption capacity of NIP in mixture solution on template molecules is weaker than in a single solution, but the adsorption capacity of MIP on template molecules is different. These results further indicate that this method has high efficiency and excellent choice for the recognition of imprinted polymers.

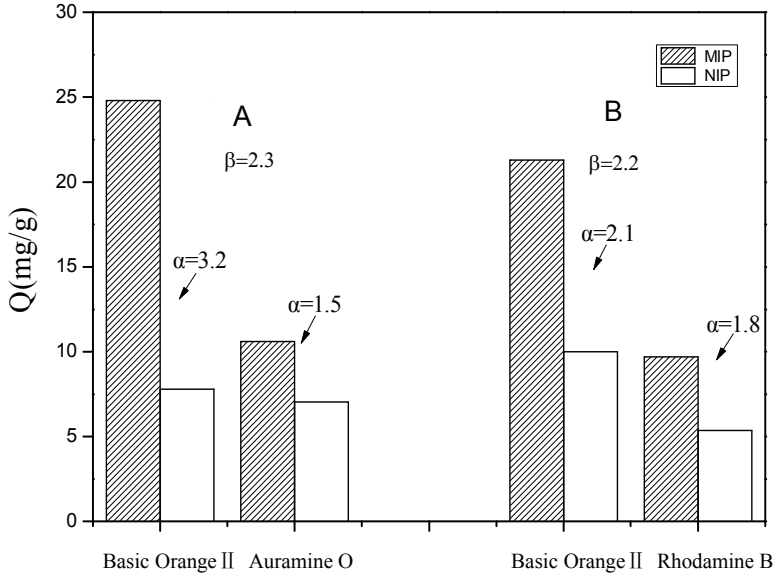


Fig.11. Adsorption capacity of MIP and NIP in competitive (A: Basic Orange II / Auramine O and B: Basic Orange II / Rhodamine B) azo dye with the initial concentration of 2.0mg/mL for each compound.

Selective adsorption performance of MISPE and C_{18} Columns

In order to confirm the superiority to selective separation and enrichment performance of MISPE column to BOⅡ, the adsorption performance of MISPE and C_{18} columns were

compared. MISPE and NISEP were prepared according to C_{18} column with same mass of filling matrix (500 mg, 6 mL) in Method 2.5. Totally 2.0 mL of 11.0 μg/mL mixture standard solution containing BOⅡ and Rhodamine B was loaded on MISPE, NISPE and C_{18} columns. The raffinate solution, cleaning fraction and eluted solution were collected. In order to ensure the simultaneous detection of BO II and Rhodamine B, the wavelength for detection was selected as 216 nm. The detection results are shown in Figure 12.

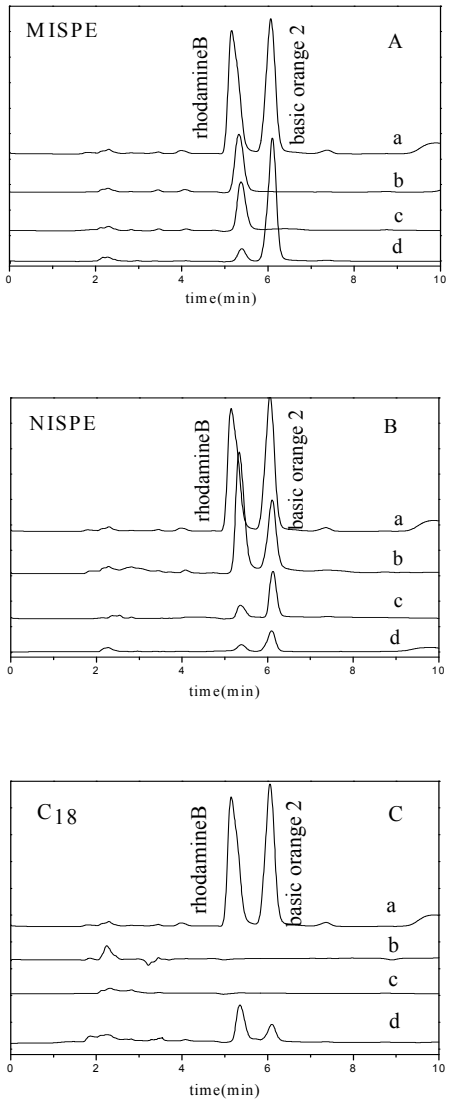


Fig.12. HPLC chromatograms of MISPE, NISPE and C_{18} SPE of standard Rhodamine B plus BOⅡ (a), solid phase extraction effluent (b), cleaning fraction (c), and elution fraction (d).

As shown in Figure 12(A), subjected to MISPE column extraction, most of Rhodamine B

together with raffinate solution and cleaning fraction was brought out the column, indicating that MISPE column did not retain Rhodamine B, while BO Ⅱ was completely retained in MISPE column. During the elution process, BO Ⅱ was basically eluted out, which indicated MISPE column had excellent selective adsorption capability to BO Ⅱ. As shown in Figure 12(B), after separating through NISPE column, most of BO Ⅱ and Rhodamine B were eluted out in raffinate solution, suggesting that BO Ⅱ and Rhodamine B cannot be retained by NISPE column and NISPE column does not have selective adsorption function on BO Ⅱ. As shown in Figure12(C), BO Ⅱ and Rhodamine B in mixture solution were completely retained on C_{18} column. Both substances in raffinate solution and cleaning fraction were not detected and only partially detected in elution fraction, indicating that C_{18} column has strong adsorption capacity on both substances in a non-selective adsorption manner. Experimental results showed that, under the SPE conditions, MISPE column is better for the selective adsorption on BO Ⅱ, and this method can be used for the separation and enrichment of BO Ⅱ.

Verification of solid phase extraction

The SPE column filled with prepared MIP can be used for the separation and enrichment of BO Ⅱ in food samples. The spiked food (dried shrimp) samples with pretreatment were loaded on MISPE column, washed with 20% ethanol, and then eluted by anhydrous ethanol. The chromatograms of each solution are shown in Figure 13.

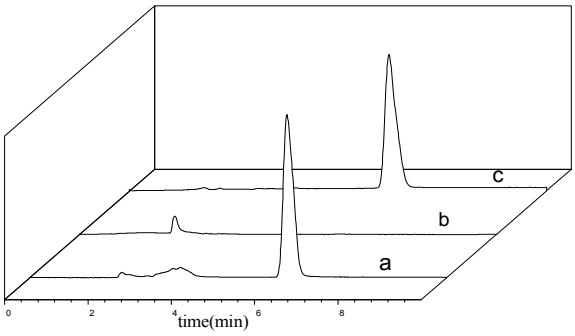


Fig.13. HPLC chromatograms of dried shrimps spiked with BO□ at the concentration of 9.0ug/g (a), cleaning fraction (b) and elution fraction (c).

As shown in Figure 13, the matrix peak of dried shrimps revealed obvious baseline

separation with chromatographic peak of BOⅡ. The matrix peak of the samples was only observed in cleaning fraction, and chromatographic peak of BOⅡ was only observed in elution fraction. After SPE and washing steps, the impurities in food samples were removed without the interference for the detection of BOⅡ. Therefore, selective SPE conditions were suitable for the separation and purification of BOⅡ.

Spiked recovery experiments and precision Test

In order to evaluate the accuracy and application of the established method, sausage, dried beancurd stick, dried shrimp and stewed tofu were used for spiked recovery experiments. As shown in Table 1, all selected samples were free of BOⅡ.

Table 1 Recovery and precision of BO□ in different spiked food samples（n=5）

Samples	Spiked (mg/kg)	Recovery (%)	RSD (%)
Sausage	5.0	80.25	1.2
Dried beancurd stick	7.0	68.43	0.71
Dried shrimp	9.0	79.00	0.15
Stewed tofu	11.0	78.45	0.86

In food samples, the recovery rates of BOⅡ were 68.43-80.25% with relative standard deviation less than 1.2%. The concentration range of the standard curve was 0.5-11 μg/mL with the correlation coefficient (R^2) of 0.9994. The detection limit of BOⅡ at three times of signal-noise ratio was 0.01 μg/mL. These results suggest that the selective separation of BOⅡ using the prepared MIP and MIP-SPE column for complex samples are reliable and can be used for the selective adsorption separation and determination of BOⅡ in food samples.

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