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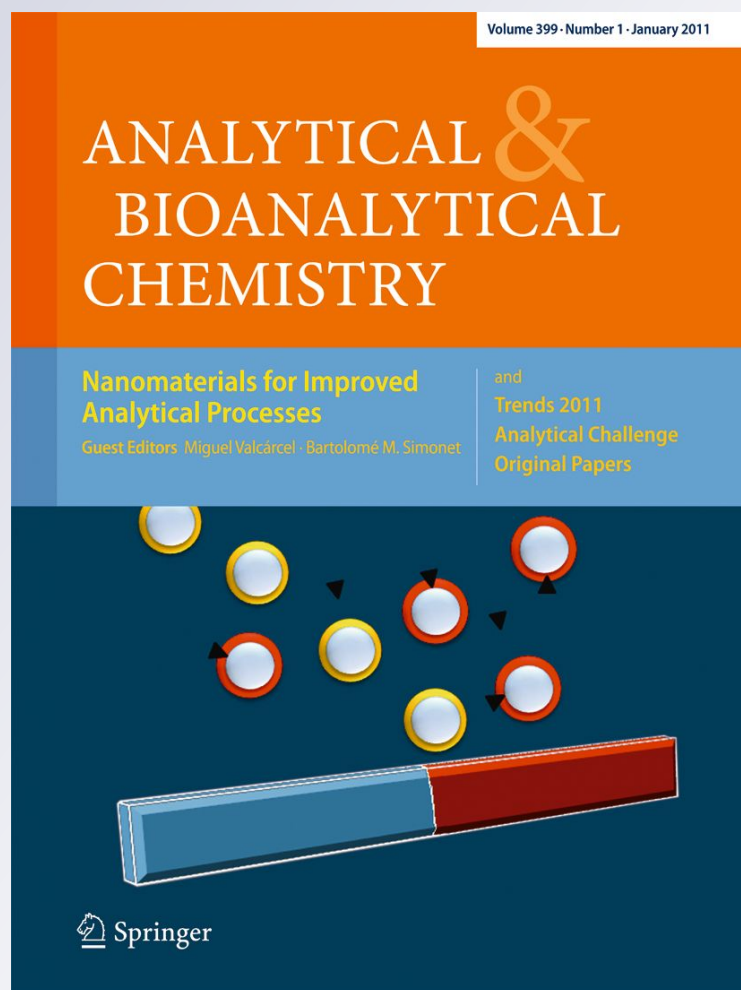
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ORIGINAL PAPER

Molecularly imprinted polymers for selective extraction of synephrine from *Aurantii Fructus Immaturus*

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Abstract In this work, molecularly imprinted solid-phase extraction (MISPE) has been used to selectively enrich, purify, or remove synephrine from *Aurantii Fructus Immaturus*. To this end, a molecularly imprinted polymer (MIP) was prepared by self-assembly from the template synephrine, the functional monomer methacrylic acid, and the crosslinker ethylene glycol dimethacrylate in 1:4:20 molar ratio. Subsequent molecular interrogation of the MIP binding sites revealed preferential structural selectivity for synephrine relative to other structurally related naturally occurring compounds (i.e. octopamine and tyramine). This selectivity was subsequently exploited to achieve substantial sample clean-up of extracts of crude *Aurantii Fructus Immaturus* and *Aurantii Fructus Immaturus* stir-baked with bran. The purity of synephrine in the extracts after MISPE represented approximately 24.21-fold enrichment of the synephrine in the untreated extracts of *Aurantii Fructus Immaturus* stir-baked with bran. High recoveries (85–90%)

from the samples proved that the method was valid for selective enrichment, purification, or removal of synephrine from *Aurantii Fructus Immaturus*.

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Introduction

Aurantii Fructus Immaturus, known in traditional Chinese medicine as "Zhishi", is the immature dried fruits of *Citrus aurantium* L. (bitter oranges) or *Citrus sinensis* Osbeck (sweet oranges); it is widely used in herbal medicine and herbal weight loss products. Bitter orange-containing dietary supplements have actually replaced ephedra-containing dietary supplements, which, in April 2004, were banned from dietary supplements by the United States Food and Drug Administration (FDA) because of association with serious adverse health effects [1]. The major compounds responsible for this activity in the herbs are adrenergic amines, for example octopamine (OCT), synephrine (SYN), and tyramine (TYR) [2, 3], which are structurally similar to norepinephrine. SYN has been found to be the major constituent of *C. aurantium* fruits and their extracts, OCT and TYR are absent or are present in low concentrations [4–6].

Although *Citrus* and its products are widely used, there are heated debates about the adverse effects and safety of *Citrus* and its primary protoalkaloid SYN. Rossato et al. [7], in a review, asserted many risks and adverse effects were associated with SYN intake. However, the review by Stohs et al. [8] stated that, on the basis of current knowledge, the use of bitter orange extract and SYN seemed to be exceedingly safe with no serious adverse

effects directly attributable to these ingredients. Because of the widespread use of *Citrus*/SYN-containing products and the relevance of its pharmacological and toxicological properties, selective enrichment or removal of SYN from extracts of *Citrus* fruit are very significant. However, most studies have focused on quantification and identification of SYN in *Citrus* fruits extracts, using different techniques such as high-performance liquid chromatography (HPLC) [3, 6, 9–15], capillary electrophoresis [16], gas chromatography [17, 18], and flow-injection analysis [19]. To purify and pre-concentrate the analytes, solid phase extraction (SPE) with a strong cation-exchange SPE cartridge [6, 18] or Waters Oasis SPE columns [17] have been used for clean-up of SYN from natural products before HPLC analysis [16]. An ion exchange process has been used to separate SYN in the leaves and fruit of *Citrus* [20], but the method is suitable for non-preferential separation and the process is complicated and the inherent lack of selectivity and consumption of solvent and time are major drawbacks. To enrich, purify, or remove SYN from a mixture, selective separation is required, because of the coexistence of compounds with similar functional groups, for example OCT and TYR (Fig. 1). Moreover, selective separation of SYN is desirable not only from the standpoint of improving pharmacological activity but also for reducing adverse effects when SYN is considered to be toxic. On the basis of these considerations, in the work discussed in this report an efficient method of separation was developed to selectively enrich, purify, or remove SYN from extracts of *Aurantii Fructus Immaturus* by molecularly imprinted solid-phase extraction (MISPE). Isolation of SYN from natural products by MISPE has not previously been reported.

MIP are synthetic material with the capability of selective molecular recognition of targeted compounds, which are called templates. MIP have specific binding sites with complementary sizes, shapes, and functional groups to the template, and involve a mechanism of interaction based on molecular recognition. These polymeric materials are generating increasing interest because of their versatility of design, molecular selectivity, durability, and reusability [21, 22]. MIP are particularly suitable in fields of scientific study that use highly selective molecular recognition processes as a key attribute, including, but not limited to, sensors, catalysis, separations, and SPE applications [23]. MIP can be used as sorbents with selectivity predetermined for a particular substance, and have been used in MISPE for

separation or clean-up of target compound present at low concentrations or in complex matrixes [24]. MISPE is an efficient approach for separation of target components from complex matrices, and may have the advantages of both MIP, with high selectivity, and SPE, with the most convenience; the technique is attracting considerable interest for separation and purification of natural products [22, 24–29]. However, MISPE has not been used for separation of SYN in *Aurantii Fructus Immaturus*.

In the work discussed in this paper, MIP were prepared by bulk polymerization, using SYN as the template, methacrylic acid (MAA) as the functional monomer, and ethylene glycol dimethacrylate (EGDMA) as crosslinker. The selectivity of the MIP and the properties of the MIP in SPE were evaluated. Separation of SYN in *Aurantii Fructus Immaturus* was carried out by MISPE.

Materials and methods

Materials and instruments

Crude *Aurantii Fructus Immaturus* and *Aurantii Fructus Immaturus* stir-baked with bran were purchased from Zhangshu Tianqitang Traditional Chinese Medicine Yinpian, Jiangxi, China. SYN hydrochloride (≥98% purity), OCT hydrochloride (≥98% purity), and TYR (≥98% purity) were purchased from the Shaanxi Sciphar Biotechnology, Shaanxi Dongke Medicine Science and Technology Incorporated Company, and Shanghai Aladdin Reagent Company, China, respectively. Methacrylic acid (MAA) and 2,2′-azoisobutyronitrile (AIBN) were purchased from the Damao Chemical Reagents, Tianjin, China. MAA was purified before use by distillation under reduced pressure. Ethylene glycol dimethacrylate (EGDMA) and Amberlite IR-120 (Na$^+$ form) cation-exchange resin were purchased from Shanghai Aladdin Reagent Company. HPLC-grade methanol was purchased from Tianjin Shield Company, Tianjin, China. Other chemicals were analytical-grade reagents; all solutions were prepared from deionized water. The surface morphology of the MIP was observed by use of an FEI Quanta 200 scanning electron microscope (SEM) under high vacuum conditions at an accelerating voltage of 20.0 kV.

SYN hydrochloride and OCT hydrochloride were converted to the free bases by passing aqueous solutions of their hydrochloride salts through an ethanol–water pre-

Fig. 1 Structures of SYN (a), OCT (b) and TYR (c)
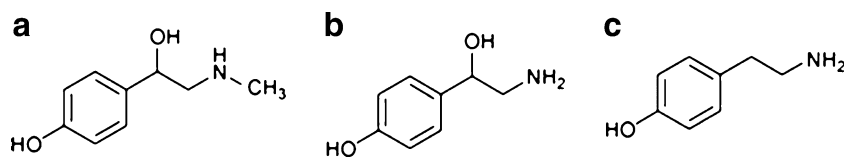
washed column of Amberlite IR-120 (H⁺ form) and subsequent elution with concentrated aqueous ammonia–ethanol (65:35) solution. The eluate was concentrated to dryness to afford the free bases as a white powders.

HPLC conditions

HPLC separation was performed on an Agilent Technologies 1100 LC system consisting of a vacuum degasser (type G1379A), a quaternary pump (type G1311A), an autosampler (type G1313A), and a diode-array detector (type G1315A). Samples were analyzed on a Sapphire-C18 column (4.6×250 mm, 5 μm particle size; Sepax Technologies) at 30 °C; the mobile phase was a 60:40 (v/v) mixture of methanol and an aqueous solution of containing 0.02% phosphoric acid, 0.02% triethylamine, and 0.1% sodium dodecyl sulfate at a flow rate of 0.8 mL min⁻¹. All analytes were detected at 224 nm and identified by comparison of retention times and UV–visible spectra with those of standards.

Preparation of *Aurantii Fructus Immaturus* extracts

Pulverized crude *Aurantii Fructus Immaturus* (0.5 g) in 68% ethanol aqueous solution (6 mL) was sonicated for 16 min with ultrasonic power of 420 W, after which the extract was centrifuged for 20 min at 5,000 rpm. An aliquot (1 mL) of the supernatant was diluted to 10 mL with acetonitrile. This solution was used for MISPE. *Aurantii Fructus Immaturus* stir-baked with bran was extracted in the same way as for crude *Aurantii Fructus Immaturus* except that an aliquot (1 mL) of the supernatant was diluted to 25 mL with acetonitrile.

MIP preparation

The MIP were synthesised as described in the literature [22] and preparation conditions were evaluated by variation of the type of porogenic solvent, the ratio of monomer to functional monomer and amount of the crosslinker. In a typical synthetic procedure, 1 mmol SYN (template) and 4 mmol MAA (functional monomer) were dissolved in 10 mL acetonitrile (porogenic solvent). The resulting mixture was sonicated for 10 min then left to stand overnight. Then 20 mmol EDMA (crosslinker) and 0.2 mmol AIBN (initiator) were added to the solution. The pre-polymerisation solution was shaken and sonicated for 10 min. The mixture was sealed and deoxygenated with a stream of nitrogen and then polymerisation was performed at 60 °C for 24 h, in a thermostat-controlled water bath, until the polymerisation was complete. Then, the rigid bulk polymer was ground to a powder with a pestle in a mortar. The particles were sieved through a 200–300 mesh

sieve. The template molecule was extracted by repeated washing with 9:1 (v/v) MeOH–AcOH until the template was no longer detected in the extraction media by HPLC. The MIP particles were then washed with MeOH to remove traces of AcOH and fines removed by repeated sedimentation in acetone. The remaining MIP particles were subsequently dried under vacuum and stored at ambient temperature. Non-imprinted polymer (NIP) was prepared under the same conditions but in the absence of template.

Static binding selectivity studies

Selectivity studies, carried out under static binding conditions, were conducted for both MIP and NIP using a constant amount of polymer (20 mg). The NIP was used to determine the extent of random, nonspecific binding resulting from interactions with the cross-linked, dispersed functional monomer. The polymer was incubated in a solution of analyte in acetonitrile or methanol. The resulting mixture was oscillated by use of a wrist-action shaker for 12 h then centrifuged at 5000 rpm for 20 min. An aliquot of the supernatant was then analyzed by HPLC, and the concentration of free analyte determined. The binding capacity (Q) of analyte on all MIP was determined as follows [30]:

$$Q = \frac{(C_0 - C)V}{m} \tag{1}$$

where Q (μmol g⁻¹) is the binding capacity, C_0 (μmol mL⁻¹) is the initiator concentration, C (μmol mL⁻¹) is the unbound concentration, V (mL) is the volume of the sample solvent, and m (g) is the mass of sorbent.

MISPE procedure

MISPE studies were conducted on SPE columns containing 100 mg of either MIP or NIP stationary phases. Dry particles of MIP and NIP (100 mg) were packed into 1.0 mL polypropylene SPE columns. The resulting SPE columns were subsequently conditioned with 5 mL of either acetonitrile or methanol, and then the SYN standard solution in acetonitrile was loaded on to the column at a flow rate of 0.2 mL min⁻¹. After loading, rinsing with 1:9 (v/v) acetonitrile–ethyl acetate was performed, and finally, elution solvent (9:1 (v/v) methanol–acetic acid) was applied at a flow rate of 0.2 mL min⁻¹ to perform complete extraction of SYN. The loading, rinsing and eluting fractions were collected and analysed by HPLC to detect the amount of SYN. The MISPE procedure for extracts of *Aurantii Fructus Immaturus* was similar to that for SYN standard solution.

Validation of the method

Calibration curve and the limits of detection and quantitation (LOD and LOQ, respectively) were determined by use of blank real sample solutions spiked with SYN in the concentration range 0.45–45 µg mL^{-1}. The sample solution (5 mL) was extracted by the MISPE procedure, the fractions collected from the elution steps were evaporated to dryness then reconstituted to 1 mL with the mobile phase, and 10 µL aliquots of these solutions were injected for HPLC analysis. LOD and LOQ were determined as the amounts for which signal-to-noise ratios were 3 and 10, respectively. The repeatability of the MISPE method was evaluated by performing five replicate analyses. Accuracy was determined by analyzing the percentage recovery of SYN from *Aurantii Fructus Immaturus* sample solutions. Samples were spiked with three different amounts of standard compound and analyzed under the previously established optimum conditions.

Results and discussion

Optimization of polymerization conditions and the MIP binding solvent

The imprinted polymer of SYN was obtained by copolymerisation of a functional monomer (MAA) and a cross-linker (EDMA) in the presence of SYN (template). It is well-known that the functional monomer plays an important role in the extraction performance for the MIP. Therefore, the effect of functional monomer types on MISPE performance was considered. For this purpose, three functional monomers MAA, methacrylamide, and methyl methacrylate were used for the preparation of the MIP. It was found that SYN failed to dissolve in the porogenic solvents in the presence of methacrylamide or methyl methacrylate, whereas it dissolved rapidly in the presence of MAA because of the ionic interaction between SYN and MAA. Moreover, MAA was selected as functional monomer because it can interact via ionic interactions with the basic secondary amine function of SYN, and the carboxylic acid group of MAA can also interact by hydrogen bonding with the hydroxyl groups of the SYN molecule [31]. To strengthen the ionic interaction between MAA and SYN and enhance the MIP selectivity, SYN hydrochloride was converted to the free base before it was used as template.

The polymerization conditions and the MIP binding solvent were optimized by binding experiments; the results, as binding capacity (Q), are summarized in Table 1. The amounts of SYN (1 mmol) and AIBN (0.2 mmol) were kept the same for all MIP preparation. As a rule of thumb, MIP are synthesised in aprotic organic solvents of low polarity, for example toluene or chloroform, in order to favour hydrogen bonding and electrostatic interactions between the template and monomer [32]. However, SYN is poorly soluble in toluene or chloroform but highly soluble in methanol or acetonitrile. Therefore, methanol (MIP1) and acetonitrile (MIP2) were selected as porogenic solvent for the investigation, because a large number of template molecules were needed for bulk polymerization. The results showed that MIP1 had a lower binding capacity than MIP2, which is largely attributed to competition between methanol and SYN for functionally important hydrogen-bonding sites, because methanol is a protic solvent. Moreover, acetonitrile is an aprotic solvent which favours hydrogen bonding between MAA and SYN. With the guarantee of sufficient solubility of the template, aprotic solvent, i.e., acetonitrile was suggested.

The effect of the MIP binding solvent (MIP2 and MIP3) on the binding capacity was obvious, when methanol (MIP2) was used. Lower binding capacity than that of MIP3 was observed, because polar protic solvents (methanol) may interfere with the binding of SYN to the MIP by competition for the formation of the non-covalent bonds.

Table 1 MIP preparation conditions and binding capacity of MIP for SYN

No.[a]	Amount of MAA (mmol)	Amount of EDMA (mmol)	Porogenic solvent	MIP binding solvent[b]	Q (µmol g^{-1})[c]	RSD (%)
MIP1	4	20	Methanol	Methanol	32.68	4.07
MIP2	4	20	Acetonitrile	Methanol	54.23	3.72
MIP3	4	20	Acetonitrile	Acetonitrile	76.82	3.15
MIP4	3	20	Acetonitrile	Acetonitrile	68.53	3.02
MIP5	6	20	Acetonitrile	Acetonitrile	63.26	3.64
MIP6	8	20	Acetonitrile	Acetonitrile	59.85	3.38
MIP7	4	10	Acetonitrile	Acetonitrile	56.84	3.53
MIP8	4	30	Acetonitrile	Acetonitrile	69.38	3.47
MIP9	4	40	Acetonitrile	Acetonitrile	61.72	3.59

[a]All MIP were prepared by adding the same amounts of SYN (1 mmol) and AIBN (0.2 mmol) in 10 mL porogenic solvent under a nitrogen atmosphere for 24 h at 60 °C

[b]MIP binding solvent is the solvent in which the static binding experiment was performed

[c]Q is the binding capacity of MIP for SYN

This is the main reason why MIP are usually used in aprotic organic solvents. Therefore, acetonitrile was used as the MIP binding solvent in the subsequent experiments.

The main function of the monomer in MIP preparation is to provide multiple recognition sites for the template molecules, and the molar ratio of template to monomer is important for sufficient self-assembly via intermolecular interactions. Therefore, the amount of MAA was investigated within the range 3–8 mmol (MIP3–MIP6). When the amount of MAA was within the range 3–4 mmol (MIP3–MIP4), an increase in the amount of the MAA resulted in acceleration of the binding capacity; when the amount of MAA was within the range 4–8 mmol (MIP4–MIP6), an increase in the amount of the MAA resulted in a decrease of binding capacity. Therefore, when 4 mmol of MAA was used, the optimum binding capacity was achieved (MIP3). These results can be explained as follows [32]. Association between the monomer and the template is governed by an equilibrium, and the functional monomers normally have to be added in excess relative to the number of moles of the template to favour formation of the complex. Therefore, excess of MAA compared with that of SYN in the pre-polymerisation mixture increased the stability of the pre-polymerisation complex between the template and the functional monomer. However, excess monomer results in an increase in the number of residual monomer groups with random orientation in the MIP and subsequent enhancement of nonselective adsorption, whereas excess template heightens the difficulty of complete template elution. Therefore, the optimum amount of MAA was 4 mmol.

The amount of the crosslinker (EDMA) is also important for MIP preparation, and will affect the degree of crosslinking and the subsequent binding properties. The effect was investigated within the range 10–40 mmol (MIP3, and MIP7–MIP9). As shown in Table 1, increasing the amount of the EDMA within the range 10–20 mmol (MIP3 and MIP7) resulted in increased binding capacity whereas increasing the amount of EDMA within the range 20–40 mmol (MIP3 and MIP8 and 9) resulted in decreased binding capacity. Therefore, the optimum amount of EDMA was 20 mmol. This is probably because increasing the amount of EDMA was beneficial to forming more binding cavities in the MIP. However, excess EDMA results in formation of a dense MIP, which will make it difficult for the SYN to enter the binding cavities.

The amount of AIBN (initiator) was considered during MIP preparation. The results showed that polymerization was incomplete and a pale yellow polymer was obtained when the amount of AIBN was very small whereas use of excess AIBN would initiate explosive polymerization. When 0.2 mmol AIBN was used, the MIP had a higher capacity factor and better imprint efficiency than that obtained using very small or excess AIBN under the same conditions. Therefore, 0.2 mmol AIBN was used during MIP preparation.

Finally, after investigation of the effects of the amount of MAA, EDMA, AIBN, type of porogenic solvent, and MIP binding solvents, the optimum polymerization conditions was achieved. Briefly, the MIP were prepared by adding 1 mmol SYN, 4 mmol MAA, 20 mmol EDMA, and 0.2 mmol AIBN to 10 mL acetonitrile under a nitrogen atmosphere for 24 h at 60 °C.

The dry MIP and NIP polymer particles were characterized by study of their microscopic morphology. From the SEM images obtained for the MIP and NIP (not shown), it was not possible to see any differences in their morphology. A porous surface could be clearly observed for the MIP and NIP, which would make it easy for the template to enter the binding cavities in the polymers through the pores, thus the template was easily bound to or eluted from the polymers.

Recognition properties of MIP and NIP

The recognition property of the MIP for SYN was studied by static equilibrium adsorption. The binding capacity Q was determined by use of a series of SYN standard solution of known concentration in methanol (Fig. 2a) or acetonitrile (Fig. 2b). As shown in the inset of Fig. 2, SYN bound to the MIP increased with increasing initial SYN concentration. The SYN bound to the MIP was more than twice that bound to the NIP in all initial concentration ranges. Most notably, the MIP had significantly preferential adsorption for SYN, which illustrated the good specificity of the MIP for the imprinted molecule. This also suggested that the imprinting process was successful. To further study the specificity, the Scatchard plot was used to discuss the binding characteristics. The Scatchard equation is [30]:

$$\frac{Q}{C} = \frac{Q_{max} - Q}{K_d} \qquad (2)$$

where K_d (mmol L^{-1}) is the dissociation constant of binding sites, Q_{max} (μmol g^{-1}) the maximum amount of apparent binding, C (mmol L^{-1}) the equilibrium concentration of SYN in binding solution, and Q is the same as in Eq. (1).

The Scatchard graph was plotted on the basis of Eq. (2) (main figure of Fig. 2). As shown in Scatchard plot of the MIP, two distinct sections appeared for the MIP isotherm, this is indicative of two groups of sites of different affinity. The affinities of the sites are related to the dissociation constants (K_d) which are calculated from the absolute value of each slope. Then, the maximum amount of apparent binding (Q_{max}) was determined from the ratio of the intercept to the slope. The binding constants of the MIP and NIP are summarized in Table 2. Irrespective of whether the binding was performed in methanol (Fig. 2a) or in
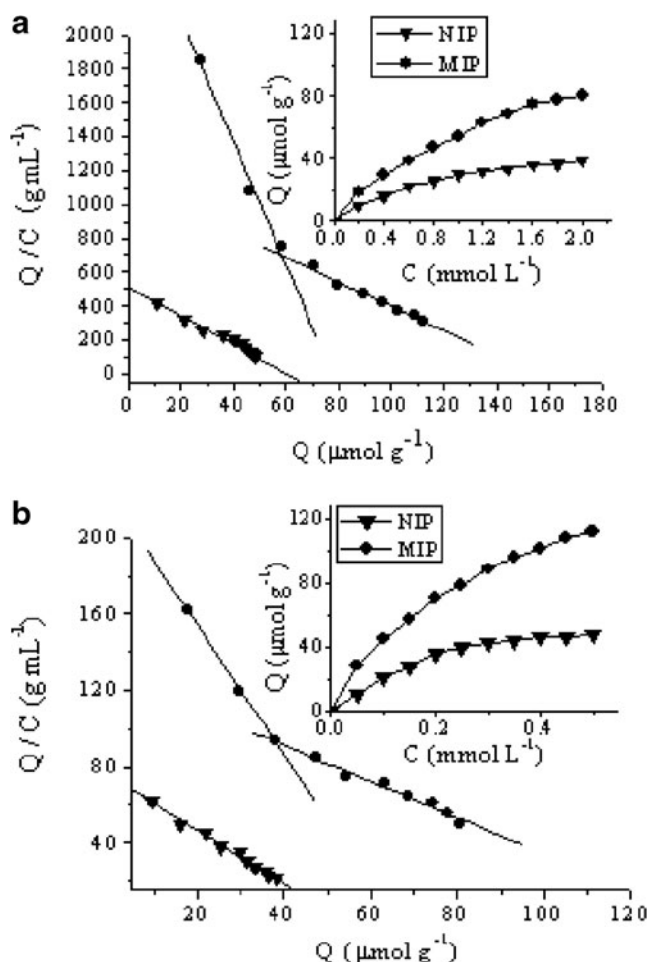
Fig. 2 Binding isotherms (*right insert*) and Scatchard plots (*main figure*) of MIP and NIP in methanol (**a**) and acetonitrile (**b**) solution

acetonitrile (Fig. 2b) solution, the experimental results showed that the MIP has higher Q_{max} in the low-affinity sites than in the high affinity sites, which are supposed to be mainly responsible for MIP specificity. The binding capacity of the MIP is the combination effect of both higher and lower-affinity sites [31, 30]. Moreover, the binding capacity of the NIP for SYN was much lower than that of the MIP, which implied that the NIP did not have the specific binding sites matching SYN and had only poor nonspecific adsorption. The specific adsorption of the MIP was achieved by imprinting was obvious.

From Table 2, we also can see that the MIP seems to have some template recognition in polar protic solvents (methanol), suggesting that proton transfer and subsequent ionic interactions may also occur through proton donation from the carboxylic acid residue of methacrylic acid to the basic secondary amine of the SYN molecule. The performance of the MIP in methanol was, however, still generally poor compared with that in acetonitrile. The reasons are as described in the section "*Optimization of polymerization*

conditions and the MIP binding solvent". Therefore acetonitrile is a better binding solvent. On the other hand, the dissociation constants (K_d) in acetonitrile solution are lower than those in methanol solution, which can, maybe, to some extent explain why the performance of the MIP in acetonitrile is generally better than that in methanol.

Recognition selectivity of MIP and NIP

To further investigate the competitive recognition coefficients of the MIP, OCT and TYR as reference compounds were chosen for measurement in a mixed solution. Both have structures similar to that of SYN and often coexist in the plant extract.

The selectivity of the MIP was evaluated by measurement of three properties—the static distribution coefficient (K_D; Eq. 3), the separation factor (α; Eq. 4), and the relative separation factor (β; Eq. 5) [30].

$$k_D = \frac{C_p}{C_s} \tag{3}$$

where C_p is the bound concentration and C_s is the unbound concentration;

$$\alpha = \frac{K_{D1}}{K_{D2}} \tag{4}$$

where K_{D1} and K_{D2} are the static distribution coefficients of the target (SYN) and competitive molecules, respectively;

$$\beta = \frac{\alpha_{MIP}}{\alpha_{NIP}} \tag{5}$$

where α_{MIP} and α_{NIP} are the separation factors of the MIP and NIP, respectively. K_D reflects the binding capacity. The bigger K_D is, the stronger the binding capacity will be. α embodies the selectivity between target molecule (SYN) and the competitor. The greater the value of α, the better the competitiveness of the binding capacity of the target molecule (SYN). The selective difference between MIP and NIP is characterized by β. The bigger β is, the stronger the selectivity resulting from molecular imprinting will be. The selectivity of the MIP is shown in Table 3. The α value of the MIP for SYN to OCT is 2.23, and that for SYN to TYR is 2.41, however, the α value of the NIP for SYN to OCT is 1.07 and for SYN to TYR is 1.05. As shown in Table 3, we know that the competitive binding capacity for SYN on the MIP is twice as large as those for the other two competitive compounds and there is not much difference among the three compounds on the NIP. This selectivity of the MIP was more than twice (β=2.08 and 2.30) as high as that of the NIP, which suggested that the imprinting process significantly improved binding selectivity for the imprinted template (SYN). Extraction of SYN by the MIP is based on its specific recognition capacity even when structurally

Table 2 Binding constants of the MIP and NIP

Sample	Binding in acetonitrile		Binding in methanol	
	Q_{max} (µmol g^{-1})	K_d (mmol L^{-1})	Q_{max} (µmol g^{-1})	K_d (mmol L^{-1})
High affinity sites in MIP	77.774	0.028	65.591	0.295
Low affinity sites in MIP	155.542	0.139	135.093	1.039
NIP	64.427	0.131	53.017	0.707

similar compounds are present. The selectivity coefficient of the MIP is larger than that of the NIP and adsorption of SYN on the MIP is specific. This demonstrates the theoretical feasibility of using the MIP of SYN as sorbent for SPE [28].

Properties of MIP for SPE

After evaluation of the efficiency of the MIP, MISPE columns were packed with MIP or the corresponding NIP, and their performance as sorbents for MISPE was compared. SYN, OCT, and TYR were chosen for the SPE experiment with the MIP as the sorbent.

Chromatograms of the analytes obtained by SPE with the MIP and the NIP are shown in Fig. 3A and B, respectively. Fig. 3A(b) shows that SYN was bound to the MIP extraction column when the standard mixture flowed through the column, whereas OCT and TYR saturated the MIP and flowed out. Figure 3A(c) shows there were large amounts of OCT and TYR but nearly no SYN in the rinsing solution after rinsing with 1:9 (v/v) acetonitrile–ethyl acetate . This shows that OCT and TYR are easily rinsed from the MIP by 1:9 (v/v) acetonitrile–ethyl acetate whereas SYN could barely be rinsed from the MIP by use of this solvent mixture. However, as shown in Fig. 3A(d), when the more strongly eluting solvent 9:1 (v/v) methanol–acetic acid was used, most of SYN was eluted from the MIP and 86.95% of the SYN was recovered. Moreover, neither OCT nor TYR was present in the eluting solution, which indicates the purity of the SYN in the mixture was significantly increased after MISPE. From Fig. 3A we can also see that the polarity of SYN is between those of OCT and TYR, yet SYN was selectively bound and retained on the MIP after rinsing with 9:1 (v/v) acetonitrile–ethyl acetate, which shows that SYN had stronger affinity for the MIP and higher imprinting efficiency than both of the

two competitive compounds, because of the selective molecular recognition.

Figure 3B(b) shows that SYN, OCT, and TYR, saturated the NIP and flowed from the column, and the ratio of SYN to OCT and TYR in the solution after SPE is similar with that in the untreated mixture before SPE. Figure 3B(c) shows that large amounts of SYN, OCT, and TYR were removed by rinsing with 1:9 (v/v) acetonitrile–ethyl acetate, the ratio of SYN to OCT and TYR in the rinsing solution is also similar to that in the untreated mixture before SPE. Finally, as shown in Fig. 3A(d), nearly no SYN was recovered by eluting the column with 9:1 (v/v) methanol–acetic acid. The results in Fig. 3B for the NIP show that SYN, OCT, and TYR had similar weak affinity for the NIP, and the NIP had no significant selectivity for enrichment of SYN.

Validation of the method

The LOD and LOQ were 0.03 and 0.12 µg mL^{-1}, respectively. The calibration curves were linear with correlation coefficients, R^2, of 0.991. The LOD and LOQ obtained in this work are low enough to enable satisfactory analysis of SYN in real samples, and match with those reported by Pellati et al. [6] in which LOD and LOQ were 0.04 and 0.13 µg mL^{-1}, respectively. The precision of the method was evaluated, and intermediate precision was obtained by repeating the SPE extraction of the same sample five times on different days; the RSD thus calculated was 2.43% and 3.36% for extracts of crude *Aurantii Fructus Immaturus* and *Aurantii Fructus Immaturus* stir-baked with bran, respectively, which showed that precision was satisfactory.

The standard addition method was used to evaluate the recovery of the MISPE process, and an extract of crude *Aurantii Fructus Immaturus* was chosen as model extract to investigate the recovery. The extract was spiked with SYN

Table 3 Selectivity of the MIP and NIP

	K_D (mL g^{-1})			α		β	
	SYN K_{D1}	OCT K_{D2}	TYR K_{D3}	α_1	α_2	β_1	β_2
MIP	523.07	234.13	216.59	2.23	2.41	2.08	2.30
NIP	171.98	161.39	164.55	1.07	1.05		

$\alpha_1 = K_{D1}/K_{D2}$; $\alpha_2 = K_{D1}/K_{D3}$; $\beta_1 = \alpha_{1MIP}/\alpha_{1NIP}$; $\beta_2 = \alpha_{2MIP}/\alpha_{2NIP}$

A

B
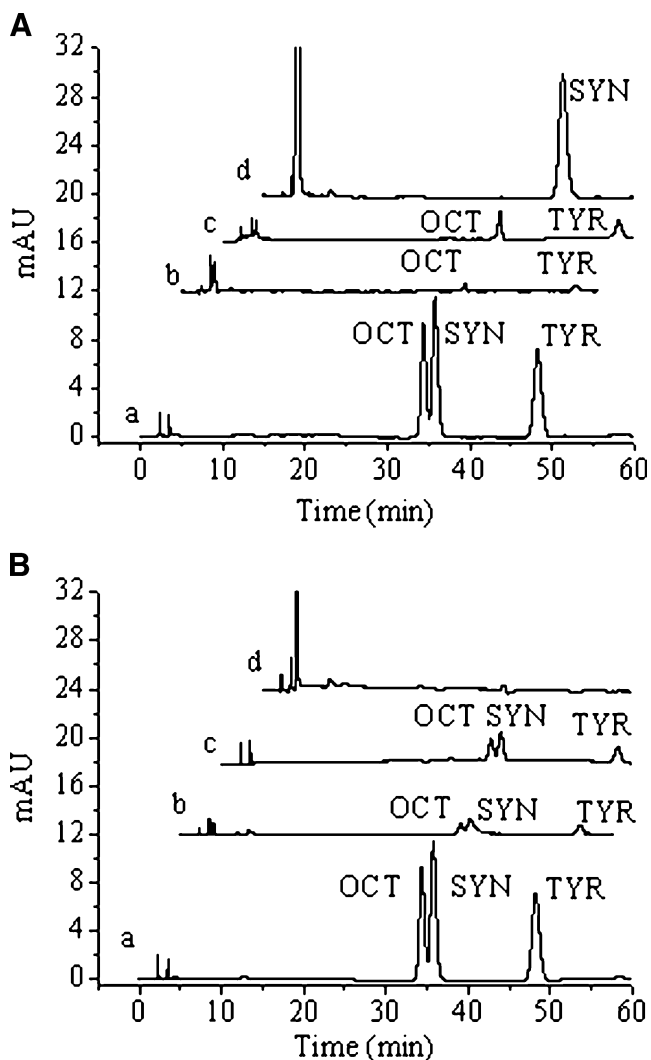
Fig. 3 Chromatograms obtained from standard mixtures of SYN, OCT, and TYR after SPE with MIP (**A**) and NIPs (**B**). (a) Untreated standard mixture before MISPE; (b) loading fraction from the MISPE column; (c) fraction rinsed from the MISPE column by use of 1:9 (*v/v*) acetonitrile–ethyl acetate; (d) fraction eluted from the MISPE column by use of 9:1 (*v/v*) methanol–acetic acid

standard solution and processed by the MISPE procedure as mentioned in the section "*MISPE procedure*" section. The results from measurement of the recovery of SYN from spiked extracts shown in Table 4 are slightly better than those reported by Pellati [6] and Andrade [18]. The results

Table 4 Recovery of SYN in extracts of crude *Aurantii Fructus Immaturus*

Concentration of the sample ($\mu g\ mL^{-1}$)	Concentration injected ($\mu g\ mL^{-1}$)	Recovery (*n*=5)	
		Mean (%)	RSD (%)
19.28	2.00	85.82	3.85
19.28	4.00	89.11	3.16
19.28	6.00	89.62	3.04

demonstrate that the MISPE process is suitable for selective enrichment, purification, or removal of SYN from extracts of *Aurantii Fructus Immaturus*.

Separation of SYN from an extract of *Aurantii Fructus Immaturus*

We used MIP in an MISPE format to examine the potential of this approach for selective enrichment and isolation of SYN from extracts of crude *Aurantii Fructus Immaturus* and *Aurantii Fructus Immaturus* stir-baked with bran. The results are shown in Fig. 4A and B, respectively. In the untreated extracts of crude *Aurantii Fructus Immaturus* (Fig. 4A(a)) and *Aurantii Fructus Immaturus* stir-baked

A

B
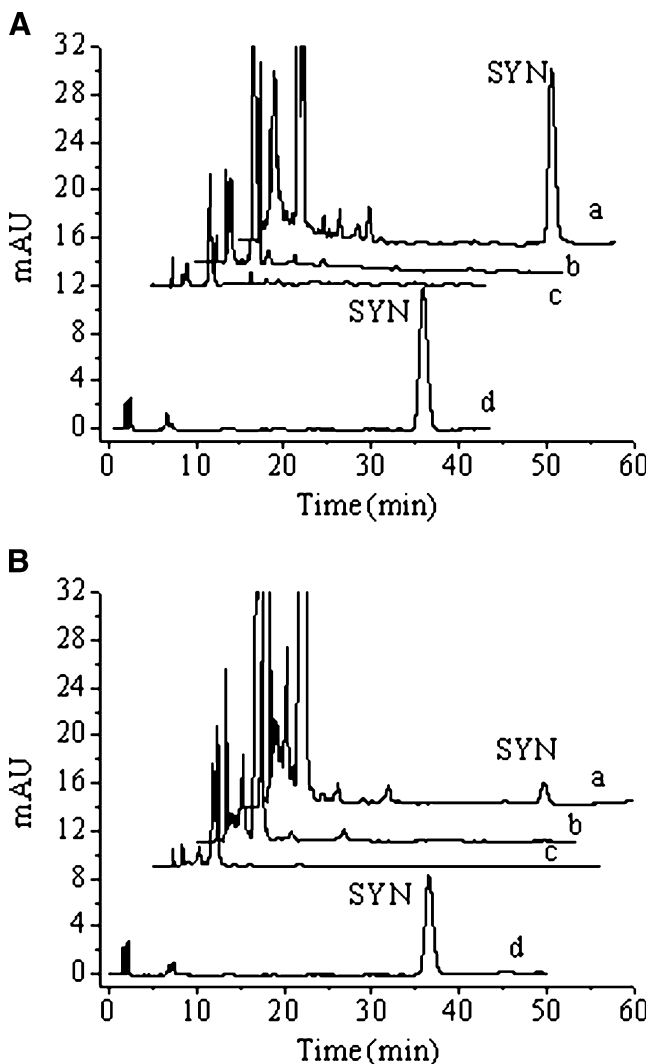
Fig. 4 Chromatograms obtained from (**A**) the extract of crude *Aurantii Fructus Immaturus* and (**B**) the extract of *Aurantii Fructus Immaturus* stir-baked with bran. (a) Untreated extract solution before MISPE; (b) loading fraction from the MISPE column; (c) fraction rinsed from the MISPE column by use of 1:9 (*v/v*) acetonitrile–ethyl acetate; (d) fraction eluted from the MISPE column by use of 9:1 (*v/v*) methanol–acetic acid

with bran (Fig. 4B(a)), OCT and TYR were undetected or present at very low concentrations, and there were many unknown components in the untreated extracts in addition to the active ingredient SYN. However, the content of unknown components in the eluting solution (Fig. 4B(d)) was greatly reduced after MISPE; 90.17% and 88.76% of SYN were recovered from the extracts of crude *Aurantii Fructus Immaturus* (Fig. 4A) and *Aurantii Fructus Immaturus* stir-baked with bran (Fig. 4B), respectively. Therefore, the MIP cartridge has achieved the desired effect that the SYN was enriched and separated from the initial extracts.

Compared with the SYN content of the untreated extract of crude *Aurantii Fructus Immaturus* (Fig. 4A(a)), that in the untreated extracts of *Aurantii Fructus Immaturus* stir-baked with bran was obviously lower (Fig. 4B(a)). It was found that the SYN content of the crude *Aurantii Fructus Immaturus* approximately was 21 times higher than that in *Aurantii Fructus Immaturus* stir-baked with bran. Therefore, the stir-baking with bran process is, maybe, an efficient approach to reducing the SYN content of crude *Aurantii Fructus Immaturus* and its extracts.

Accordingly, MISPE using 3 g MIP as sorbent was used for the extraction of SYN from the extract of *Aurantii Fructus Immaturus* stir-baked with bran. After MISPE, the eluting solution was evaporated to dryness under vacuum and the purity of the residue (MISPE extract) was analyzed by HPLC. The purity of SYN in the extracts after MISPE was indicative of approximately 24.21-fold enrichment of the SYN from the untreated extracts before MISPE, with high recovery of SYN (87.50%), i.e. an increase in the purity to 8.23% from 0.34% (on a mass and mass-normalized basis). Therefore, the MIP clearly resulted in significant sample clean-up of the extracts of *Aurantii Fructus Immaturus* or enrichment of SYN.

Conclusion

In this work, a molecularly imprinted solid phase extraction (MISPE) procedure was developed for selective extraction of SYN from extracts of *Aurantii Fructus Immaturus*. SYN-imprinted polymers were synthesized using SYN as template, MAA as functional monomer, and EGDMA as crosslinker. Optimum MIP preparation conditions for enhanced recognition properties of MIP toward SYN were obtained. The obtained polymer had good selectivity and high binding capacity for SYN. MIP were then used as sorbent in SPE to purify SYN from the standard mixture of SYN, OCT, and TYR, and from extracts of crude *Aurantii Fructus Immaturus* and *Aurantii Fructus Immaturus* stir-baked with bran. The purity of SYN in the extracts after MISPE represented approximately 24.21-fold enrichment of the SYN from the *Aurantii Fructus Immaturus* stir-baked

with bran. The high recoveries (86–90%) from the samples proved the method was suitable for selective enrichment, purification, or removal of SYN from different samples of *Aurantii Fructus Immaturus*.

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