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Molecularly imprinted polymer for selective extraction and simultaneous determination of four tropane alkaloids from *Przewalskia tangutica* Maxim. fruit extracts using LC-MS/MS

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A class-specific molecularly imprinted polymer (MIP) for selective extraction and preconcentration of four tropane alkaloids has been successfully prepared by precipitation polymerization using anisodine (ASD) as template, methacrylic (MAA) as the functional monomer, trimethylolpropane trimethacrylate (TRIM) as crosslinker and acetonitrile as the porogen. The performance of the MIPs and the non-molecularly imprinted polymers (NIPs) were evaluated in terms of selective recognition capacity, adsorption isotherms and adsorption kinetics. The results indicated that MIPs exhibited significant specific recognition toward TAs with a large adsorption capacity. To testify the feasibility of MIP in real sample preparation, the obtained MIPs were applied as the selective sorbent for solid- phase extraction of four TAs in *Przewalskia tangutica* Maxim.. Under optimized conditions, a rapid, convenient and efficient method for the determination of four TAs in *Przewalskia tangutica* Maxim. fruit samples was established by class-specific MIPs based MISPE coupling with high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) . The method gave excellent recoveries (82.1–102%) and precision (RSDs < 5%, n = 5) for *Przewalskia tangutica* Maxim. fruit extracts spiked at three concentration levels (50, 100, 200 μg L^{-1}). The results demonstrated that the TAs in the extracts could be separated and purified through MIP-SPE from *Przewalskia tangutica* Maxim..

1. Introduction

Przewalskia tangutica Maxim., belonging to the plant family Solanaceae, is an important and rare medicinal plant found in the Tibetan Plateau of China. The roots, seeds and the entire grass of *Przewalskia tangutica* Maxim. is widely used as a common Tibetan medicine for the treatments of gastrointestinal spasm pain, diphtheria, anthrax, and other diseases due to its analgesic, spasm modulation, pesticidal, and anti-inflammatory effects.[1-2] A large number of studies have shown that of the tropane alkaloids (TAs) in *Przewalskia tangutica* Maxim., anisodine (ASD), scopolamine (SPM), anisodamine (ASM) and atropine (ATP) (Fig. 1A), are the key active substances. [3-4] Due to their biological activity, a great deal of methods for the separation and purification of TAs from complex matrices have been investigated by radioimmunoassays,[5] capillary electrophoresis (CE), [6] thin layer chromatography (TLC)[7-8] and high performance liquid chromatography (HPLC).[9-10] However, even when using these methods, the determination of TAs in the pharmaceutical preparations process often requires several complicated sample pretreatment procedures, such as extraction, separation, and concentration. Although these pre-treatment operation processes are essential, they are time-consuming and could not obtain selective recognition during the extraction. Furthermore, the low abundance, complex composition and matrix interferences in real samples increased the difficult for the extraction and determination of TAs. There is, therefore, a need to develop selective and efficient enrichment methodology for tropane alkaloids in natural plant matrices.

Molecularly imprinted polymers (MIPs) are synthetic porous materials that possess tailored binding sites with memory of the shape, size and functional groups of the template molecules. These sites can selectively recognize a guest molecule or related analogous compounds.[11-12] MIPs exhibit many outstanding advantages including high selectivity, [13] chemical and physical robustness, [14-15] reusability, and low cost of preparation.[16-17] Due to their outstanding advantages, MIPs have been applied as molecular-recognition elements in areas such as chemical sensors,[18] food analysis,[19] pharmaceutical analysis,[20] drug delivery,[21] catalysis,[22] and solid-phase extraction (SPE).[23] In particular, MIPs used as adsorbents of SPE are widely involved in the concentration and enrichment of drug molecules, and the extraction of active components from complex natural matrices.[24-27] Theodoridis et al.[28] reported the MIPs for hyoscyamine prepared by a typical bulk polymerization method as off-line SPE, and they applied it to the selective extraction of scopolamine from biological samples.

In fact, TAs often exist as a mixture of several compounds such as anisodine, scopolamine, anisodamine and atropine in *Przewalskia tangutica* Maxim. and other medicinal plants.[29] So during the

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processing of these raw materials, how to obtain four TAs simultaneously is a valuable and interesting strategy. In recent years, the wide potential of MIP-SPE in studies on natural products, the selective extraction of not only a target compound but also structural analogues, has been demonstrated.[30-32] To the best of our knowledge, MIPs as sorbent have not been described for the simultaneous enrichment of four types of TA compounds, nor has a group-selective MIP-SPE system been reported for the extraction and isolation of such TAs from natural herbs.

In this study, we designed a novel monodispersed class-specific MIP in a micro-spherical bead format for SPE of four TAs. The aim of this work was to provide a simple and effective technique to concentrate the TAs existed in the traditional Chinese medicines, and then realize its accurate quantification by LC-MS/MS on the basis of high sensitivity. To achieve this objective, we prepared a uniformly sized MIPs for TAs by a precipitation polymerization method with anisodine as the template molecule. In our work, characteristics of the MIPs were evaluated by HPLC in terms of selective recognition capacity, adsorption isotherms and adsorption kinetics. Further, the obtained MIPs was applied as the selective sorbent for solid- phase extraction of TAs from *Przewalskia tangutica* Maxim. fruits. As a result, simultaneous extraction and determination of four TAs in natural herbs was successfully achieved by LC–MS/MS based on an optimized MISPE procedure without complicated pre-treatment.

2. Experimental

2.1 Reagents and materials

Certified standards of anisodine hydrobromide, Scopolamine hydrobromide, anisodamine hydrobromide, atropine sulfate and rutin (RU) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Dried Tibetan herb, *Przewalskia tangutica* Maxim., was purchased from Jinzhu Tibetan Medicine Corp. Ltd (Tibet, China). Methacrylic acid (MAA), trimethylolpropane trimethacrylate (TRIM), α, α′-azobisisobutyronitrile were obtained from Sigma Aldrich (St. Louis, MO, United States). Formic acid, acetonitrile and methanol were HPLC grade and were purchased from Merck (Darmstadt, Germany). Ultrapurified water was obtained from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA). Dichloromethane, chloroform, acetic ether, ligarine, carbon tetrachloride, isopropanol, n-hexane and acetic acid of analytical grade were obtained from the Chemical Reagent Company (Beijing, China). Three-milliliter-capacity SPE cartridges and the corresponding frits were purchased from Agilent (Beijing, China). All the other chemicals were of analytical grade and used without further purification.

Stock solutions (1000 μg mL^{-1}) of each standard were prepared by dissolving the appropriate amount in methanol. The stock solutions were stored at 4 °C in the dark for no longer than 3 months. Working standard solutions were prepared by diluting the stock standard solutions with methanol or acetonitrile.

The mature fruits of P. tangutica were collected from the county of Lhari Zong in the Tibet Autonomous Region of China in 2013. The plant materials were identified by Prof. Nyima Tashi, Plant taxonomist, Tibet Academy of Agricultural and Animal Husbandry Sciences, china. Voucher specimens were deposited at the department of traditional Tibetan medicine in the institute of veterinary and animal husbandry, Tibet Academy of Agricultural and Animal Husbandry Sciences, China.

2.2 Instrumentation and operation parameters

Ultraviolet spectra were recorded using a Nanodrop 2000C (Thermo Scientific, Massachusetts, USA). Scanning electron microscopic (SEM) measurements were performed with a Hitachi S-4800 (Hitachi Limited, Tokyo, Japan) field emission scanning electron microscope at an acceleration voltage of 5 kV. The samples were coated with a thin gold film before analysis. Fourier transform infrared (FTIR) spectra in were recorded by KBr discusing a Philips PU9800 spectrometer (Philips Analytical, Cambridge, UK).

The adsorption selectivity and the adsorption capacities of the MIPs were assessed by HPLC. The HPLC system consisted of a Waters 2695 Alliance HPLC system (Waters Corporation, Milford, MA, USA) equipped with a PDA detector operating at 195 nm. Chromatographic experiments were carried out using a C18 column (Agilent Eclipse, XDB; 4.6 mm×150 mm, 5 μm) operating at 25 °C. The mobile phase was 0.5% (v/v) potassium dihydrogen phosphate (A) and methanol (B) (75:25, v/v). The pH of buffer solutions was adjusted with a pH meter (GLP 22, Crison, Barcelona, Spain).

The determination method of four TAs in *Przewalskia tangutica* Maxim. fruit was validated by LC–MS/MS based on an optimized MISPE procedure .Chromatographic system consisting of (Agilent Technologies, Palo Alto CA, USA) coupled to an API 2000 mass spectrometer (Applied Biosystems, AB Sciex, Foster City, CA, USA) equipped with a Turbo Ion Spray (ESI) interface. The instrument was supported by performed with the AB Sciex Analyst 1.4.2 software (Applied Bioscience) for control System, data acquisition, and analysis.

2.3 Spectroscopic analysis of interaction between template and monomers

The interactions between the ASD template and the functional monomers in solution prior to polymerization were characterized using UV spectrophotometry. A series of solutions were prepared with a fixed concentration of ASD and various amounts of MAA, 4-vinylpyridine (4-VP) and 4-vinylbenzoic acid (4-VA) in acetonitrile. After equilibrating the solutions for 30 min, the changes in absorbance and the difference absorption spectra for these solutions were recorded, with pure ASD solution in acetonitrile serving as a reference.

2.4 Preparation of the ASD-imprinted and non-imprinted polymers

The preparations of monodispersed bead-shaped MIP particles for ASD and NIP particles were performed by precipitation polymerization. ASD (40 mg, 0.1 mmol) and MAA (34 μL, 0.4 mmol) were dispersed in 50 mL of acetonitrile by ultrasonic vibration for 30 min. TRIM (319 μL, 1 mmol) and AIBN (50 mg) were then dissolved into the above solution. The mixture was degassed with N_2 for 10 min and the flask was kept in a rotamantle with mild stirring. The temperature was ramped from room temperature to 60 °C over a period of 1 h and then kept constant at this temperature for 23 h. After polymerization, the polymer particles were washed with methanol and collected by centrifugation at 13,000 rpm and 4 °C for 10 min. Finally, the template was extracted by washing the polymer beads extensively with methanol/acetic acid (80/20, v/v) in a Soxhlet extractor. The MIPs were washed until ASD was not detected using HPLC. The residual acetic acid was removed from the MIPs by washing with methanol followed by several washings with ultrapure water. NIP was prepared following exactly the same procedure described above, but in the absence of the ASD template.

2.5 Evaluation of MIP performance

To evaluate the adsorption performance of the ASD-MIPs, both static and dynamic adsorption experiments were carried out in acetonitrile solution to confirm the rebinding and recognition performances of the MIPs and the NIPs. For various concentrations

of ASD over the range 2.5–100 μg mL^{-1}, 1 mL solutions containing 2.5 mg of polymer (MIPs) were equilibrated at room temperature using mechanical shakers for 2 h. Following equilibration, the tubes were centrifuged at 10,000 rpm for 10 min and the supernatants were withdrawn under nitrogen, reconstituted with methanol and then measured the concentration of free ASD. The amount of the template rebound (Q, ug g^{-1}) was calculated for each polymer using Eq. (1):[33]

$$Q = (C_o - C_e)\ V/m, \quad (1)$$

where C_0 (mg mL^{-1})and C_e (mg mL^{-1}) are the initial and final concentrations of the incubated solution, respectively, V (mL) is the volume of the solution and m (mg) is the mass of the polymer added.

In addition, the imprinting efficiency (Ie) was introduced to evaluate the adsorption capability. This was obtained from the ratio of Q_{MIP} to Q_{NIP} according to the following Eq. (2), where Q_{MIP} and Q_{NIP} represent the respective adsorption quantities:

$$I_e = Q_{MIP}/Q_{NIP}. \quad (2)$$

Meanwhile, the dynamic adsorption experiment was carried out as follows: 5 mg of the ASD-MIPs and NIPs were added to one mL of four mixed and rutin standard solution at a concentration of 60 μg mL^{-1}. One milliliter of loading solvent varying for different periods of time (1–24 h) was carried out.

The subsequent Scatchard analysis of the polymers was evaluated. The binding isotherms obtained through the equilibrium experiments were transformed to linear equations by use of the Scatchard Eq. (3).

$$\frac{Q}{C_e} = \frac{Q_{max} - Q}{K_d}, \quad (3)$$

where Q is the amount of TAs bound to the polymer at equilibrium, C_e is the concentration of free analytes in solution (instead of the initial concentration in solution), Q_{max} is the apparent maximum number of binding sites and K_d (μM) is the apparent dissociation constant.

The selective recognition capacity was evaluated using SPM, ASM and ATP with the best MIP, these compounds being structurally similar to ASD. For this purpose, 5 mg of the polymer as well as the structural analogues were separately equilibrated with 60 μg mL^{-1} of ASD for 2 h using the same procedure as discussed above. All the solutions above were centrifuged and the supernatants were analyzed by HPLC to determine final concentrations and indicate the extent of adsorption. The amounts of the structural analogues bound were quantified using a 5-point calibration curve for the concentration range 5–1000 μg L^{-1}.

2.6 Application of MISPE to Przewalskia tangutica Maxim. fruit extracts

2.6.1 preparation of extracts of *Przewalskia tangutica* Maxim. Fruit samples. 1 g of *Przewalskia tangutica* Maxim. fruit powder sample was weighed into a vial and soaked in 3.0 mL of ammonia solution followed by vortexing for 2 min. Subsequently 30 mL of acetonitrile were added and the sample was agitated on a shaker for 30 min. Other solvents such as chloroform, acetic ether, dichloromethane-isopropanol (3:1, v/v) were also used to compare with acetonitrile. The samples were then centrifuged at 8000 rpm for 5 min and the collected supernatants were defatted and enriched. The procedure was repeated three times until the extract became clear. Finally, the supernatant was evaporated on a rotary evaporator, dissolved in methanol (1.0 mL) and then purified by following the optimized MISPE procedure.

2.6.2 Conditions for MISPE. 100 mg of the MIP beads dispersed in 2.0 mL acetone were loaded into a 3.0 mL SPE cartridge, the beads being retained between two polyethylene frits. Prior to each extraction, the cartridges were conditioned with 3.0 mL of acetonitrile. One milliliter of sample solution was loaded onto the MIP or NIP cartridge and then washed with different solvent and finally the TAs were eluted with selected eluent. The eluate was analyzed after reconstitution by methanol and the recoveries of the analytes were calculated.

Table 1 MS/MS parameters for the determination of four TAs
*Quantitative ion.

Compounds	Parent mass (m/z)	Daughter mass (m/z)	Declustering potential (V)	Collision energy (eV)
ATP	290.2	124.1 * 93.0	69	36 47
SPM	304.1	155.9 * 138.2 120.8	36	29 25 34
ASM	306.4	140.1* 122.0	70	36 41
ASD	320.0	156.0* 138.1 119.1	69	27 33 35

2.6.3 Validation of MISPE method in real samples. Przewalskia tangutica Maxim. extracts were spiked at three concentration levels (50, 100, 200 μg L^{-1}) in the MIP and NIP cartridges, respectively. The purified solutions were analyzed by HPLC after reconstitution in methanol and the recoveries of TAs were calculated.

2.7 LC–MS/MS analysis

One milliliter extracts eluted from the MISPE cartridge were filtered through nylon filter and injected into the LC-MS/MS system.

Chromatographic separation was carried out with a reversed-phase analytical column (Agilent Eclipse XDB-C18 column, 150×2.1 mm, 5 μm), kept at 30°C. A gradient elution was performed from 0.1% formic acid (v/v) in water (A) and 0.1% formic acid (v/v) in methanol(B). A starting gradient at 10.0% of mobile phase B with a hold of 10.0 min and then increased to 90% in 10.0 min with a hold of 7.0 min and then to 10% again, with a flow rate of 200 μL min^{-1} and a injected volume of 5 μL.

Electrospray interface (ESI) was operated in the positive mode, and the typical ESI parameters used were as follows: ion spray voltage (IS), 5500 V; atomization air pressure(GS1), 25 psi; auxiliary gas pressure (GS2), 40 psi; curtain gas (CUR), 40 psi; ion source temperature (TEM), 500 °C; entrance potential (EP), 10 V; and collision cell exit potential (CXP), 4 V. The MRM transitions, collision energy (CE), and declustering potential (DP) are summarized in Table 1.

3 Results and discussion

3.1 Interaction mechanism between template molecule and functional monomer

Precipitation polymerization is the most widely used technique for obtaining spherical particles. The basic principle of this approach is that, when the polymeric chains growing in solution reach a certain critical mass, they precipitate from solution.[34] Apart from the inherent advantage of avoiding crushing and sieving steps, this technique leads to uniform particle size, which results in higher performance in comparison to bulk polymerization.[35] Therefore, in this study, a series of ASD-MIPs were synthesized by precipitation

polymerization.

When the template and functional monomers form complexes in solution, the strength of these complexes is reflected in the affinity and selectivity of the imprinted polymer.[36-37] Therefore, studies on the intermolecular interactions between ASD and the functional monomers prior to polymerization would be important for probing the recognition mechanism of the imprinted polymers and the binding between ASD and the functional monomer. Because the cross-linker and initiator have less influence on the interactions between the template and the functional monomers, spectrophotometric studies were performed in the absence of TRIM and AIBN. In this experiment, the interactions between ASD and MAA, 4-VP and 4-VA in the pre-polymerization stage were estimated via spectrophotometric assay, respectively. As shown in Fig**.** 3A, in the presence of MAA, a new absorption peak with relatively high absorption occurred at 196 nm. However, on the addition of 4-VP or 4-VA to the ASD solution, no additional absorption peak was observed, rather a band with very low absorption appeared. Also, Fig**.** 3B shows that the maximum wavelength shift in the absorption of ASD was 1.0 nm from its original value (195 nm) in the former system where the molar ratio of ASD to MAA was 1:4, instead of 1:6 and 1:8. In contrast, for ASD with 4-VP or 4-VA, no clear change in the wavelength of maximum UV absorption of the template was observed. A possible explanation for this finding was the role of π–π stacking interactions which might have had an even larger impact than electrostatic interactions between ASD and MAA. As can be seen from Fig**.** 1B, there are two hydroxyl groups, a tertiary amine, an oxirane and a benzene ring group in the ASD molecule. MAA was selected as the functional monomer because it was conducive to hydrogen bond or ionic bond interactions in the porogen prior to polymerization.[38] Electrostatic interactions due to the tertiary amine and carboxy moieties of MAA, which provide hydrogen bonding interactions from –OH and the π–π stacking from ethylenic linkage to ASD, might result in a tight configuration between the pre-polymers. The MAA monomer shows not only hydrogen bonding and electrostatic interactions, but also π–π stacking, which result in many more combining sites which provide the hydrogen bonding interactions with 4-vinylpyridine (4-VP) and 4-vinylbenzoic acid (4-VA). Consequently, in the following experiments for the preparation of the ASD-MIP., MAA was used as the functional monomer and the molar ratio of template to monomer was 1:4.

3.2 Morphological analysis

Scanning electron microscopy was used to illustrate the detailed morphology of the ASD-MIP and its corresponding NIP. As shown in Fig**.** 4, a highly ordered and porous structure was produced and the pore diameters of the MIPs were mostly regular spheres with a size of 400–800 nm, whereas in the case of the NIPs, the spheres had a rough surface and were of irregular volume and having slightly smaller diameters in the range of 100–400 nm. Additionally, SEM images permitted visualization of the beads and the images for the MIPs showed uniformly shaped and spherical beads with a smooth surface. It has been shown that most recognition sites on MIPs tend to be evenly distributed on the surface of the supporting core.[39] This is clearly beneficial for achieving fast adsorption and desorption of the four analytes and would ensure adequate performance of the MIP in terms of dissociation, extraction and determination of trace TAs by MISPE-HPLC.

To further characterize the ASD-MIP beads, FT-IR spectra for MIP, NIP and MAA were assessed. As shown in Fig. 5, the functional groups corresponding to the prominent absorption peaks were identified. The peak at 1737 cm^{-1}, resulting from the C=O stretching vibration, is indicative of MAA and TRIM having formed an ester bond of the fatty acid. Three intense peaks at 2976 and 1470 cm^{-1} were attributed to the stretching vibration and flexing vibrations of methyl groups, respectively. No peaks were detected at wavenumbers higher than 3000 cm^{-1}, indicating an absence of hydroxyl groups and the existence of fatty acids in the MIP particles. It can, therefore, be concluded that methanol in the elution step interacted with the MIP system by esterification. Simultaneously, the presence of residual C–C bonds in the MIP coating was indicated by a minor absorption peak at around 1690 cm^{-1}; this was crucial for the chemical bonding between the initial MIP monolayer and subsequent layers during the repeated coating procedure.[40] What's more, the absorption frequency of CO in the ester bind of ASD at 1740 cm^{-1} was shifting downward to 1720 cm^{-1}, accompanied with the decrease of its absorbance. It could be speculated that there might be another H-bonding formed between CO and $–NH_2$ in the ASD and MAA mixture. All the FTIR spectral features were consistent with the MIPs having been prepared successfully. The characteristic peaks in the FTIR spectra of the MIPs underwent a wavelength shift relative to that for the NIPs, confirming that the templates and monomer had undergone successful pre-polymerization.

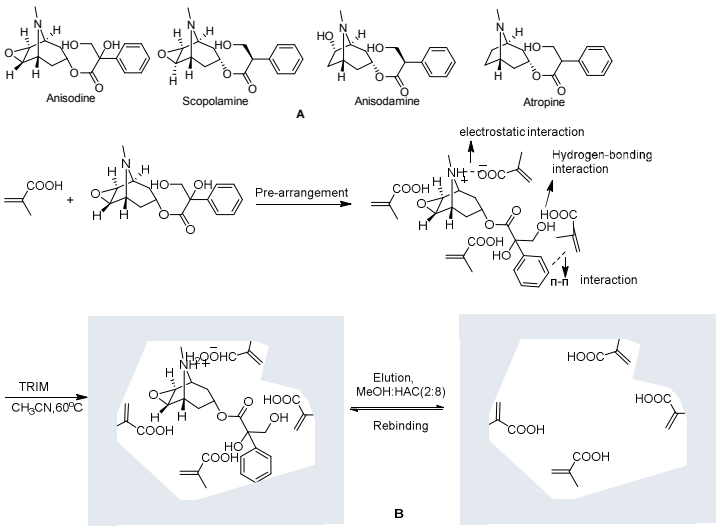

Fig. 1 Molecular structures of the four tropane alkaloids (A) and Schematic illustration of the fabrication mechanism of the ASD-MIPs (B)

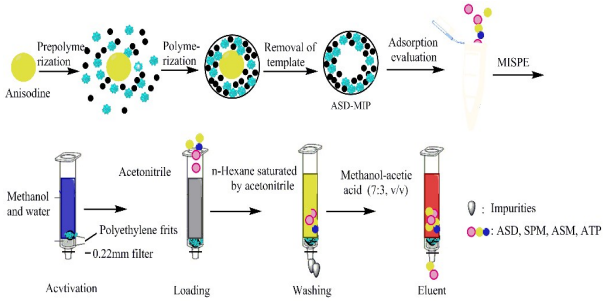

Fig. 2 Schematic diagram of MISPE procedure for selective adsorption of four tropane alkaloids

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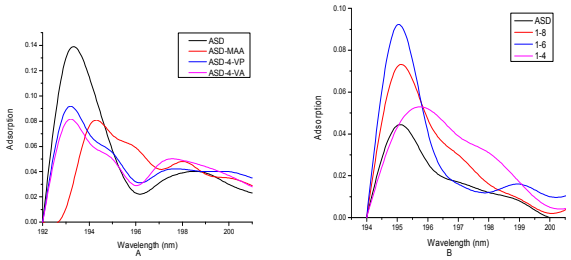

Fig. 3 The UV spectra of ASD with different monomers (A) and ASD with MAA in different ratios (B)

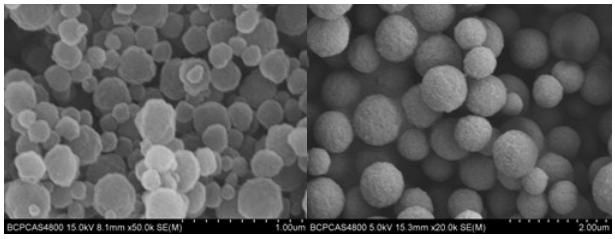

Fig. 4 Scanning electron micrographs of (A) NIPs and (B) ASD-MIPs

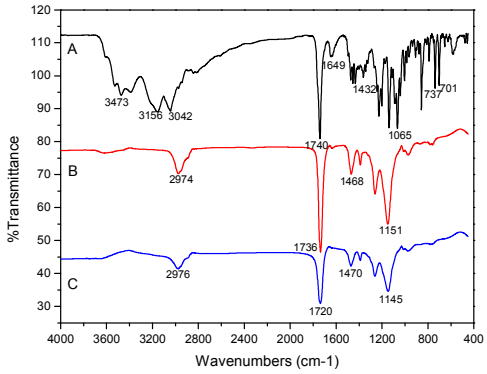

Fig. 5 Infrared (KBr pellet) spectra of ASD (A), NIPs (B) and MIPs (C)

3.3 Evaluation of adsorption performance

The binding isotherms of ASD on the MIPs and NIPs are shown in Fig. 6A. The adsorption capacity of the polymers increased with the concentration of ASD from 2.5 μg mL^{-1} to 100 μg mL^{-1}. Fig. 6A clearly shows that the adsorption capacity of the MIPs was much higher than that of the NIPs when the concentration was greater than 60 μg mL^{-1}. Thus, it would be reasonable to assume that the MIPs have specific binding sites for TAs in the polymer network. However, the MIPs prepared in this work had the highest binding affinity for ASM and the lowest for the template ASD. This may be a reflection of the richer hydrogen bond content of the ASM which would provide a stronger non-specific affinity due to the presence of more abundant hydroxyl groups. These results will need to be confirmed by independent study.

Acetonitrile was used as the mobile phase for the MIPs and the NIPs with I_e values ranging between 1.42 and 3.58 for the different tested TAs. Rutin was adsorbed to verify the selectivity of MIPs to tropane alkaloids and non-analogue. Results showed clearly from Fig. 6B that the prepared MIPs possess favorable specificity and selectivity characteristics for the four analytes, especially for template ASD (I_e = 3.58) and ATP (I_e = 2.25). The values of SPM and ASM were slightly lower, being 1.49 and 1.42, respectively. But MIPs showed barely selectivity for rutin.

A curve can be obtained for the Scatchard plot rather than a straight line when the polymer has more than one class of binding sites.[41] In the case of one ligand (ASD) which has two types of binding sites (one with high and the other with low selectivity), two straight lines would be generated with different slopes according to the Scatchard equation. As concluded by Castell etc.,[42] occupation of the binding sites with the highest affinity prevailed in the low concentration range, and binding with the lowest affinity sites occurred in the high concentration range. All the other binding sites, which exhibited a variety of binding affinities between these two extremes, were situated in the intermediate area. Therefore, the values for Q_{max} and K_d were apparent values, which can be used for comparative purposes rather than absolute values when considering the assumptions made in the Scatchard approach.[41] However, they do indicate the presence of different binding sites on the MIP given that a curved plot was observed, especially when compared with the NIP, which is always represented by a straight line.

The different slopes for the MIP and NIP signify different binding mechanisms. The linear regression equations for the linear regions were: $Q/C_e = -65.181Q + 411.55$ (R^2 = 0.9942) and $Q/C_e = -4.1722Q + 57.306$ (R^2 = 0.9701). From the slopes and intercepts of the fitted lines, the values for K_d and Q_{max} were 0.053 μmol L^{-1} and 6.3 μmol g^{-1}, and 0.83 μmol L^{-1} and 13.73 μmol g^{-1}, respectively.

As displayed in Fig. 7, The NIP curve shows a linear slope, indicative of one type of binding site possessing low selectivity. The MIP, however, displayed different slopes, which indicates the presence of heterogeneous binding sites. Thus the presence of the two plots with differing slopes represent binding sites with higher affinities (MIP high) and lower affinities (MIP low). The binding sites with high selectivity have a lower dissociation rate and are present in smaller amounts than those retained in the low-selective sites. The overall affinity of the MIP toward the target analyte is higher compared with the NIP, as displayed by the K_d values.

3.4 Optimization of the MISPE protocol and selectivity

To obtain sample purification and preconcentration by the MIP in SPE, the experimental conditions for the MISPE cartridges, such as the loading, washing and elution steps were investigated and optimized.[43] It is theorized that the formation of monomer-

template complexes will be enhanced when the analytes are loaded as solutions in the polymerization solvent (porogen). In this study, the results showed that the recoveries by MISPE for the analytes were higher with acetonitrile as the loading solvent rather than when solvents such as methanol and dichloromethane were used.

3.4.1 The composition of the washing solvent. The wash step was crucial to maximizing the specific interactions between the analytes and the binding sites and to simultaneously decrease nonspecific interactions so that matrix components of the sample could be discarded.[44-45] Thus, after loading 1.0 mL of spiked sample into the cartridge, 1.0 mL of MeOH-H_2O (1:1, v/v), acetonitrile (ACN), H_2O, dichloromethane (DCM), chloroform (DMK), ethyl acetate (EA), carbon tetrachloride (CCl_4), petroleum ether (PE) and acetonitrile saturated n-hexane as wash solvents were investigated, respectively (Fig. 8). The results showed that MeOH-H_2O (1:1, v/v) was effective in removing the impurities from the sample matrix, but most of the analytes were also simultaneously co-eluted. The studies indicated that 1.0 mL was a suitable volume of wash solution to efficiently remove the interferences from the sample

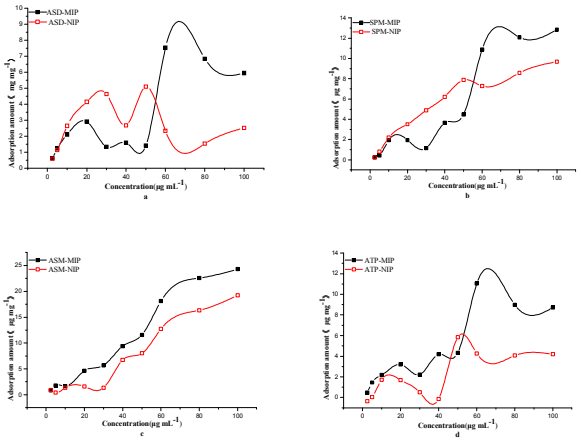

Fig. 6A Adsorption isotherm of MIP and NIP for atropine and its analogues （a. ASD, b. SPM, c. ASM, d. ATP）

C_o of four tropane alkaloids were 2.5-100 μg mL^{-1}, V= 4mL, m= 5mg, t= 12h, T= 25 °C, solvent is acetonitrile.

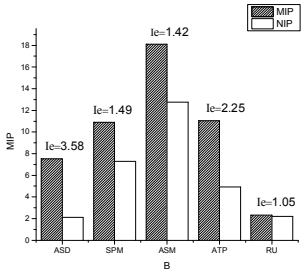

Fig. 6B The specificity adsorption of ASD-MIP and ASD-NIP for RU, ASD and analogues

Co of four tropane alkaloids were 2.5-100μg.mL^{-1}, V=4mL, m=5 mg, t=12h, T ＝25℃ , solvent is acetonitrile

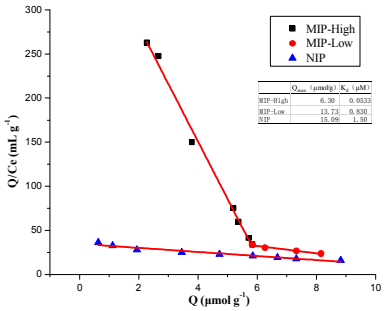

Fig. 7 Scatchard plots for the MIP and NIP isotherms.
The ratio of the bound to free concentration of anisodine solution (mL g^{-1}) is plotted *vs* the amount of anisodine bound to the MIP or NIP (μM g^{-1}).

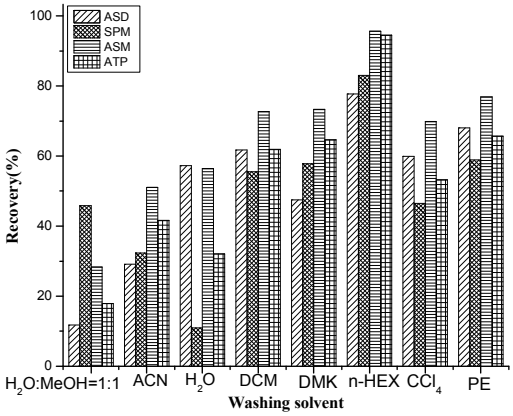

Fig. 8 Effect of different washing solvents on analyte recoveries

matrix. Considering recoveries, purification effects and economic factors, 1.0 mL of acetonitrile saturated n-hexane was chosen as the optimum wash solution.

3.4.2 Elution solvent selection. The template and analogue molecules bind with the MIPs mainly by specific binding, hence, strongly polar solvents such as acetic acid (HAC)-methanol (MeOH) were used to achieve quantitative elution of the analytes, this being consistent with elution in the MIP preparation procedure. To obtain a satisfactory recovery of the TAs, the elution step was also optimized. Comparing HAC-MeOH at two differing strengths, i.e., 2:8 v/v and 3:7, v/v, respectively, the latter was found to elute all analytes with use of only 2 mL of solution. Considering the cost, further experiments were concerned with identifying the optimum volume and volume ratio for the mixed solvent.

3.4.3 The reproducibility of the MIP cartridges. Repeatability studies were performed using three MIP cartridges prepared from different batches of polymer by loading 1 mL of acetonitrile spiked with the TAs at 10 μg mL^{-1} on each of the MIP cartridges and then determining the analytes in the eluates by HPLC. Under the optimized conditions mentioned above, mean recoveries for the extraction efficiencies of ASD, SPM, ASM and ATP were 82.2%, 88.8%, 77.4% and 82.8%, respectively, and the RSDs were less than 3.7%. In addition, investigations demonstrated that at least 30 cycles could be performed with the same cartridge without any significant effects on MISPE performance, indicating the good repeatability and reusability of the ASD-MIP packed cartridges.

3.5 Validation of the Method

The accuracy and precision of the LC-MS/MS method was assessed by analyzing the *Przewalskia tangutica* Maxim. fruit samples fortified with TAs at six concentration levels ranging from 5 to 1000 μg L^{-1} (n = 3). Under the optimal conditions, liner regression calibrationcurves are shown in Table 2. Good linearity in response (R^2 > 0.99) was obtained for the four analytes over the concentration range examined and no significant differences (confidence level 95%) were observed between the slopes of the standard addition curve and that obtained after pre-concentration of the standard solutions of the TAs (data not shown), confirming the absence of matrix effects under the optimized MISPE conditions. The limits of detection (LODs) based on signal-to-noise ratios of 3.0 were 0.52 – 2.12 μg L^{-1}. The intra-day precisions, evaluated as the RSDs, were less than 1.84%. The results demonstrated that the precision of this method was acceptable.

The amounts of ASD, SPM, ASM, ATP that were detected from the fruit samples by MISPE were 30, 80, 158, 261 μg g^{-1}, respectively, after the test liquid diluted one ten times. The accuracy of the MISPE method was estimated by evaluating tissue samples spiked with TAs at the concentration of 50, 100 and 200 μg L^{-1}. As can be seen from Table 3, the mean recoveries of MISPE for the four TAs ranged from 82.1% to 102% with RSD % values ranging from 2.12% to 5.00%, which demonstrated that the MISPE served as an efficient SPE sorbent for the extraction and enrichment of TAs in herbal products. The NIPs cartridge, in contrast, gave low found concentration compared with the MIPs, which reflects a relatively poor selectivity for adsorption of the TAs.

Table 2 Parameters of the LC-MS/MS Method

Analytes	MISPE-LC-MS/MS Method		
	Linearity (μg L^{-1})	LOD (μg L^{-1} n=5)	RSD (%)
ASD	5-1000	1.00	1.33
SPM	5-1000	2.12	1.84
ASM	5-1000	1.05	1.56
ATP	5-1000	0.52	1.12

Table 3 Accuracy of the MISPE- LC-MS/MS method for four tropane alkaloids in *Przewalskia tangutica* Maxim. fruit extracts

Cartridge	ASD				SPM		
	Spiked concentration (μg L^{-1})	Found concentration (μg L^{-1})	Recovery (%) (n = 5)	Precision (RSD, %) Intra-day (n = 5)	Found concentration (μg L^{-1})	Recovery (%) (n = 5)	Precision (RSD, %) Intra-day (n = 5)
NIP	0	18.0	—	4.89	35.0	—	3.99
MIP	0	30.0	—	2.59	80.0	—	2.12
	50	71.0	82.1	4.14	127	94.5	4.37
	100	131	101	3.52	176	96.2	4.28
	200	229	99.5	3.12	275	97.5	4.89

Table 3 Accuracy of the MISPE- LC-MS/MS method for four tropane alkaloids in *Przewalskia tangutica* Maxim. fruit extracts (continued)

Cartridge	ASM				ATP		
	Spiked concentration (μg L^{-1})	Found concentration (μg L^{-1})	Recovery (%) (n = 5	Precision (RSD, %) Intra-day (n = 5)	Found concentration (μg L^{-1})	Recovery (%) (n = 5)	Precision (RSD,%) Intra-day (n = 5)
NIP	0	75.0	—	4.95	78.0	—	3.19
MIP	0	158	—	3.64	261	—	3.08
	50	207	98.1	4.08	312	102	3.55
	100	254	96.4	2.52	360	99.9	4.22
	200	355	98.5	5.00	456	97.4	3.99

3.6 Analytical application

In order to further verify the practicability of this method, the extracts of *Przewalskia tangutica* Maxim. fruits were tested as real samples. After 30 ml acetonitrile was applied to samples, the extracted solution was evaporated to dryness and redissolved in methanol (1.0 mL) and then purified by following the optimized MISPE procedure. The chromatograms for selected samples are shown in Fig. 9. The TAs mixtures (the reference materials) were analyzed by LC-MS/MS and the results showed that the signal sequence was in the order ASD, SPM, ASM and ATP. Fig. 9D reports the chromatogram of the eluting fraction, showing the prominent peak for TAs. Setting the eluting results against those of the crude extraction before MISPE (Fig. 9A), the signals for ASD were barely sufficient for identification purposes due to matrix interference. The chromatogram for the load step showed good absorbility and wash step for the MISPE cartridge shows that the signal intensities were very low at the retention times corresponding to that of the TAs (Fig. 9C) and the SPM relatively loss large. After purifying the samples with the MIP-SPE column, elution step performed well-defined peaks demonstrated excellent purification of the method (Fig. 9D). In summary, experimental results revealed that the TAs in *Przewalskia tangutica* Maxim. fruit extracts were extracted and purified successfully at the same time using the MISPE column.

4. Conclusions

In this study, a class-specific molecularly imprinted polymer for the selective extraction of four tropane alkaloids (TAs, ASD, SPM, ASM, ATP) was successfully prepared using MAA as functional monomer and acetonitrile as porogen, with a 1:4:10 molar ratio of ASD to MAA and crosslinker TRIM. The ordered porous polymers showed high absorption capacity, good class-specific selectivity and reproducibility. The prepared MIPs served as a MISPE adsorbing material to selectively enrich and determine tropane alkaloids (TAs) in *Przewalskia tangutica* Maxim. fruits coupled with LC-MS/MS. MISPE demonstrated to be a useful tool for the determination and quantification of four TAs in *Przewalskia tangutica* Maxim. fruits, allowing a simple, fast, and specific sample extraction. Therefore, it was hoped that the proposed method would be used for the highly selective separation and fast determination of tropane alkaloids in other medicinal plants and biological samples.

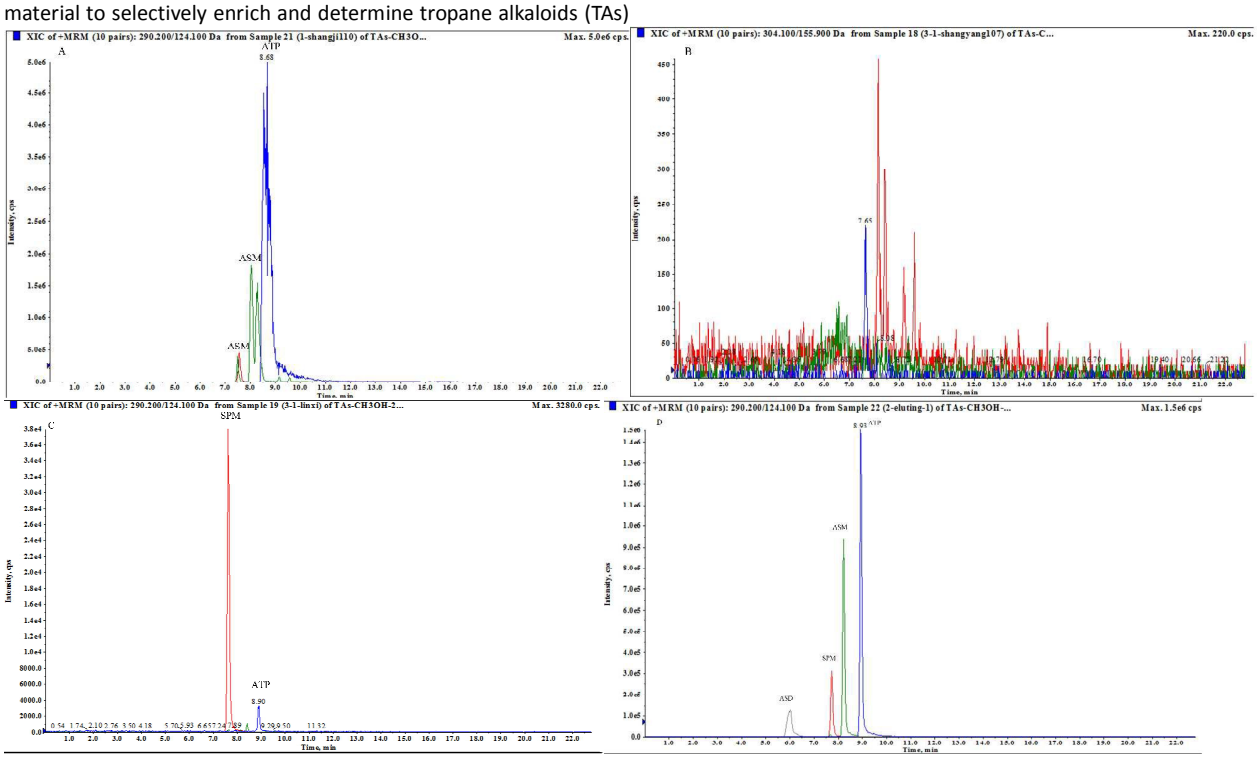

Fig. 9 Chromatogram of TAs in the samples. (**A**) Chromatogram of crude extract of *Przewalskia tangutica* Maxim. fruits. (**B**) Chromatogram of loading solution from MIP-SPE column. (**C**) Chromatogram of washing solution from MIP-SPE column. (**D**) Chromatogram of eluate from MIP-SPE column

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