

Article

A Novel Mesoporous Multi-Template Molecularly Imprinted Polymer for Selective Separation and Determination of *Panax notoginseng* Saponins Simultaneously in Biological Samples

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Abstract: The feasible, reliable and selective multi-template molecularly imprinted polymers (MT-MIPs) based on SBA-15 (SBA-15@MT-MIPs) for the selective separation and determination of the trace level of ginsenoside Rb₁ (Rb₁), ginsenoside Rg₁ (Rg₁) and notoginsenoside R₁ (R₁) (*Panax notoginseng* saponins, PNS) from biological samples were developed. The polymers were constructed by SBA-15 as support, Rb₁, Rg₁, R₁ as multi-template, acrylamide (AM) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as cross-linker. The new synthetic SBA-15@MT-MIPs were satisfactorily applied to solid-phase extraction (SPE) coupled with high performance liquid chromatography (HPLC) for the separation and determination of trace PNS in plasma samples. Under the optimized conditions, the limits of detection (LODs) and quantitation (LOQs) of the proposed method for Rb₁, Rg₁ and R₁ were in the range of 0.63-0.75 ng mL⁻¹ and 2.1-2.5 ng mL⁻¹, respectively. The recoveries of R₁, Rb₁ and Rg₁ were obtained between 93.4% and 104.3% with relative standard deviations (RSDs) in the range of 3.3-4.2%. All results show that the obtained SBA-15@MT-MIPs could be a promising prospect for the practical application in the selective separation and enrichment of trace PNS in the biological samples.

Keywords: multi-template molecularly imprinted polymers; SBA-15; *Panax notoginseng* saponins; separation and determination; solid-phase extraction

1. Introduction

Panax notoginseng (Burk.) F. H. Chen (*P. notoginseng*) is a species of the genus *Panax*, family Araliaceae, which has been officially recorded in USP Herbal Medicines Compendium and China Pharmacopeia [1,2]. The radix and rhizome of *P. notoginseng*, known as Sanqi or Tianqi in East Asian countries, is one of the primary herbs in natural products. *Sanqi* has wide-ranging pharmacological effect such as anti-inflammation [3], cardioprotective effect [4], anticarcinogenic effect [5], anti-atherosclerotic [6] as well as antioxidant [7], etc. The major bioactive constituents responsible for these pharmacological effects are extensively recognized as the *Panax notoginseng* saponins (PNS), among which ginsenoside Rb₁ (Rb₁), ginsenoside Rg₁ (Rg₁) and notoginsenoside R₁ (R₁) are three main components and thus generally considered as quality control markers in the manufacturing of *Sanqi* related preparations [2,8]. The content and their ratios of PNS in *Sanqi* are also strongly correlated to its quality and efficacy [9]. In the current scenario, a few studies on the toxic effects of PNS due to

unstable components, impurities in prescriptions and PNS over dose have appeared during clinical treatments [10-12]. Until now, various methods have been already proposed to assay PNS, including high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS) [13], gas chromatography-mass spectrometry (GC-MS) [14], micellar electrokinetic chromatography (MEKC) [15] and enzyme-linked immunosorbent assay (ELISA) [16]. However, because of multi-components, extremely low concentrations of PNS and the complex nature of the matrices in complex samples [17], it is almost impossible to directly identify PNS without time-consuming pre-treatment. Currently, different attempts have been proposed and investigated, which included liquid-liquid extraction (LLE) [18], ultrafiltration (UF) membrane [19], microdialysis [20], etc. Although these pre-treatment techniques have been applied to extract a wide variety of compounds, these protocols involve some disadvantages, such as severe interference, low enrichment efficiency and limited sensitivity, etc. Therefore, it is highly desirable to establish a feasible, selective and sensitive analytical method for extraction, enrichment and quantification of major active components in body fluids to ensure the safety, efficacy and stability of clinical application of PNS.

Molecular imprinting technology (MIT), often described as a method of making a molecular lock to match a molecular key, is a technique for the creation of molecular imprinting polymers (MIPs) with tailor-made recognition sites complementary to template molecules in shape, size, and functional groups [21]. The recognition sites are generated by a process that involves copolymerization of functional monomers and cross-linkers around template molecules [22]. Interestingly enough, templates are removed from the polymers rendering complementary binding sites capable of subsequent template molecule recognition [23,24]. Due to their unique features of high specificity, mechanical strength and resistance against organic solvent, high pressure and temperature, MIPs have gained enormous utility in electrochemical sensor [25], solid-phase extraction [26], and biomimetic catalysis [27], etc. Regrettably, traditional MIP bulks exhibit some disadvantages, including incomplete template removal, poor site accessibility to target species and irregular shape, which is bound to affect the rebinding and selective recognition of target molecules. The surface imprinting technology can overcome the above problems effectively. Recently, mesoporous silica has been proven to be an excellent matrix material that possesses high hydrothermal stability, tunable pore sizes, ordered mesoporous structure, and large specific surface areas [29], which can significantly enhance the adsorption capability of porous adsorbents. Compared to other mesoporous materials, SBA-15 exhibits larger pore size and thicker pore wall [30]. Based on SBA-15, the present study was focused on synthesizing novel MIPs endowed with higher specific surface area, more accessible binding site and faster mass transfer rate.

In our initial studies, we synthesized highly selective single-template MIPs based on mesoporous materials for selective separation and quantitative analysis of natural products in biological specimens [31,32]. As we all know, the single-template MIPs could not exhibit high affinity and selectivity for congeneric compounds [33]. Furthermore, the single-template MIPs are not efficient in the case of recognition of various targeted analyses simultaneously from complex samples [34]. In addition, physically mixing individually imprinted polymers are also not feasible as synthesizing several polymers require considerable time and efforts [35]. Actually, by using several targets/species as templates, different classes of species can be extracted, separated, assayed and detected simultaneously, which can increase their utility and expand their potential application [35]. To circumvent the fundamental defects of single-template MIPs, multi-template MIPs (MT-MIPs) prepared with two or multiple templates, which can simultaneously absorb a group of structurally analogues, are highly desirable for sustainable development. However, there is no publication to till date that reports the simultaneous separation, enrichment and detection of PNS based on MT-MIPs.

In this study, we prepared highly sensitive and selective polymers as a novel and inexpensive sorbent for the isolation and enrichment of PNS in biological samples. The MT-MIPs based on SBA-15 (SBA-15@MT-MIPs) were synthesized using R₁, R_{b1}, R_{g1} as multi-template, AM as functional monomer, EGDMA as cross-linker, and EtOH as porogen. The resulting products were characterized by TEM, FT-IR, TGA, N₂ adsorption-desorption analysis. Adsorption properties of obtained SBA-

15@MT-MIPs were investigated, which included adsorption isotherm, adsorption kinetics and selective recognition. In addition, a molecularly imprinted solid-phase extraction (MISPE) procedure coupled with HPLC was successfully developed to isolate and detect trace amounts of PNS in rat plasma samples, which indicated that the novel SBA-15@MT-MIPs can provide a proper example of an analytical method for quality control and support pharmacological and clinical studies of PNS.

2. Materials and Methods

2.1. Chemicals and reagents

Ginsenoside R_{g1} (R_{g1}), ginsenoside R_{b1} (R_{b1}), notoginsenoside R₁ (R₁), ursolic acid (UA), oleanolic acid (OA) were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Pluronic P123, tetraethoxy silane (TEOS), tetrahydrofuran (THF), ethylene glycol dimethacrylate (EGDMA) and 4-vinylpyridine (4-VP) were purchased from Sigma-Aldrich (Steinheim, Germany). Acrylamide (AM), methacrylic acid (MAA), toluene (TOL), acetonitrile (ACN), acetic acid (HAc) and 2,2-azobisisobutyronitrile (AIBN) from Sinopharm (Shanghai, China). 3-Methacryloxypropyltrimethoxysilane (MPS) was obtained from Aladdin (Shanghai, China). ACN of HPLC grade was prepared from Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was produced using a Millipore water purification system (Merck Millipore, Germany) and used for the entire experiment. All other chemicals used in this study were of the analytical reagent grade.

The powder of *Panax notoginseng* was donated by the Department of Pharmacognosy, Nanjing Medical University (Nanjing, China).

2.2. Apparatus

The FT-IR analysis was conducted in the form of KBr pellets and using a Fourier Transform Infrared (FT-IR) spectrometer (Bruker, Switzerland) in a range of 500–4000 cm⁻¹. Ultraviolet-Visible (UV-Vis) absorbance detection was achieved by a Shimadzu UV-2100 spectrophotometer. Heating magnetic stirrer (C-MAG HS 7, IKA, Germany) was applied during the preparation. The morphologies and structures of polymers were observed by a scanning electron microscope (SEM, FEI Quanta 200, USA) and a transmission electron microscope (TEM, JEM-2100, JEOL, Japan). A shake culture box (ZHLY-180, China) was applied during the binding experiment. N₂ adsorption-desorption analysis was carried out at 77 K using a Micromeritics ASAP 2010 system. Thermogravimetric analysis was conducted on a thermogravimetric analyzer (TGA, Pyris 1 DSC, Perkin-Elmer, USA) with a heating rate of 20°C min⁻¹ from 25–700°C under a stream of N₂ gas (200 mL min⁻¹). HPLC analysis was performed with a Shimadzu (Japan) system comprising LC-20AT pump, SPD-M 20A detector, CTO-20A column oven and HW-2000 chromatographic work station. The structural identification of target compounds was performed using an Agilent 1200 liquid chromatograph coupled with an Agilent 6410B Triple Quad mass spectrometer.

2.3. Synthesis of SBA-15 support and modified SBA-15

2.3.1. Synthesis of SBA-15 carrier

The SBA-15 was synthesized according to the previous literature [32]. In brief, to 250 mL round-bottomed flask, 2.0 g of P123 was mixed with 60 g HCl (2.0 mmol L⁻¹), 2.0 g of HAc and 15 g of H₂O under vigorous stirring at 40°C. Subsequently, 4.25 g TEOS was added into the abovementioned solution drop by drop under vigorous stirring for 24 h at 40°C and the mixture was transferred into Teflon-lined autoclaves and heated at 100°C for 48 h. Finally, the resulting precipitates were filtered and washed with distilled water, and then dried at ambient temperature. The organic templates were removed by calcining in air for 6 h at a heating rate of 5°C min⁻¹ up to 550°C.

2.3.2. Preparation of vinyl modified SBA-15

The surface of the as-prepared SBA-15 was modified by MPS according to our previous report [31]. Briefly, 500.0 mg SBA-15, 10 mL MPS, and 50 mL anhydrous TOL were placed into a 100 mL three-necked flask and stirred. Then, the mixture solution was degassed with N₂ for about 10 min followed by heating the mixture to 55°C for 24 h. Finally, the modified SBA-15 (SBA-15-MPS) containing vinyl terminal group was centrifuged, washed with TOL and MeOH successively and dried under vacuum at 45°C for 24 h.

2.4. Imprinting at the surface of modified SBA-15

The SBA-15@MT-MIPs were prepared by surface molecular imprinting technique, as shown in Figure 1. Here, a facile free-radical polymerization method was employed. Prior to polymerization, 0.2 mmol PNS (the ratio of R_{b1} to R_{g1} to R_l was 44:43:13 presented in *P. notoginseng*) and functional monomer were dissolved in 30 mL of solvent, which was incubated 4 h at ambient temperature for pre-polymerization to prepare the prearranged solution. Then 0.1 g SBA-15-MPS was dispersed in 20 mL of solvent by ultrasonic vibration. The prearranged solution, cross-linker (EGDMA) and initiator (AIBN) (the weight was about 5% of the total mass of templates, functional monomers and cross-linkers) were added to the above solution and mixed. The mixture was deaerated by N₂ for 20 min and carried out in a shake culture box at 60°C for 24 h. After the reaction, the SBA-15@MT-MIPs were centrifuged and washed successively with MeOH to remove all absorbed oligomers and unreacted monomers. Finally, the SBA-15@MT-MIPs were washed repeatedly with MeOH-HAc (9:1, v/v) to remove the templates until no PNS in the supernatant was detected using a UV spectrophotometer. The resulting imprinted polymers were repeatedly washed with MeOH until the supernatant was neutral, and then dried in a vacuum at 40°C for 24 h. The multi-template non-imprinted polymers based on SBA-15 (SBA-15@MT-NIPs) were also fabricated and purified under identical condition except that the templates were omitted.

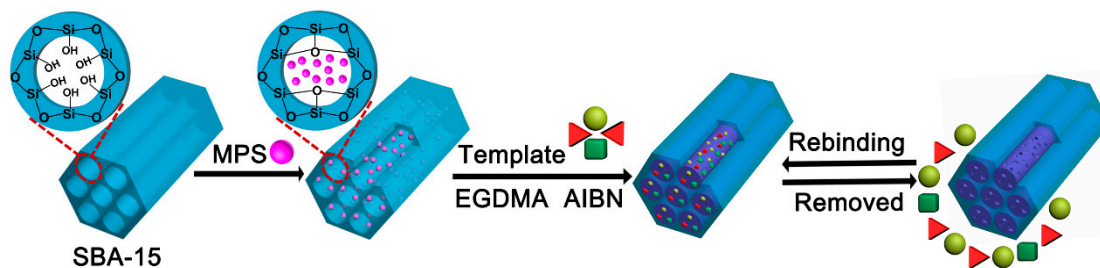


Figure 1. Schematic representation for the preparation of the SBA-15@MT-MIPs.

2.5. Evaluation of the binding ability of SBA-15@MT-MIPs

To evaluate the adsorption performance of SBA-15@MT-MIPs, the isotherm, kinetics and selective adsorption experiments were carried out. All experiments were repeated three times in parallel. In all experiments, 20 mg polymers were dispersed in a 5 mL adsorption solution. The mixture was incubated on a shaker at 200 rpm for a period of time, and then filtered using a 0.22 µm microporous membrane. The maximum absorbance of PNS at about 204 nm before and after adsorption was measured by UV-Vis spectrophotometer and the corresponding concentrations were calculated through the calibration curve. The adsorption capacity (Q , µmol g⁻¹) of the template bound to the polymer was calculated by the following equation (1):

$$Q_e = \frac{(C_0 - C_e)V}{M} \quad (1)$$

In order to investigate the recognition performance of adsorbents for target molecules, an equilibrium adsorption experiment was studied at various concentrations of PNS ranging from 0.5 to 4.0 mmol L⁻¹. The mixture was kept shaking for 80 min at ambient temperature.

To investigate the adsorption rate of the obtained SBA-15@MT-MIPs, an adsorption kinetic experiment was conducted. The adsorption time was changed at regular intervals from 0 to 80 min, while the PNS solution was kept at a concentration of 3.0 mmol L⁻¹.

To measure the specificity of SBA-15@MT-MIPs, UA and OA were adopted as structural analogues to compare with PNS. A selective adsorption experiment was investigated using solutions of R₁, R_{g1}, R_{b1}, UA and OA at a concentration of 3.0 mmol L⁻¹ and incubation time of 60 min. The imprinting factor (IF) was used to evaluate the specific recognition property of the polymers towards the targets and structural analogues, which was calculated by equation (2):

$$IF = \frac{Q_{MIP}}{Q_{NIP}} \quad (2)$$

Here Q_{MIP} and Q_{NIP} are the adsorption capacities of the template and/or structural analog on SBA-15@MT-MIPs and SBA-15@MT-NIPs, respectively.

To investigate the stability and reusability of SBA-15@MT-MIPs, six adsorption-desorption cycles were conducted by using the same SBA-15@MT-MIPs. The adsorption procedure was the same as the binding experiment. The concentration of PNS solution and incubation time was 3.0 mmol L⁻¹ and 60 min, respectively. Elution of PNS from SBA-15@MT-MIPs was performed using MeOH-HAc (9:1, v/v) and MeOH in turn.

2.6. Separation and determination of PNS in real samples

2.6.1. Preparation of plasma samples

50.0 g of *P. notoginseng* powder was weighted accurately and placed into a 500 mL round-bottom flask and 400 mL of 70% EtOH was added. The mixture was refluxed for 4 h at 80°C. The extraction was repeated two additional times, and the combined extracts were concentrated to about 10 mL under vacuum and then diluted to 50 mL with physiological saline. Ten male SD rats (250-280 g) were fed with standard laboratory food and water ad libitum except for fasting 12 h prior to experiment. The extract solution was orally administered to rats at the doses equivalent to 10 g kg⁻¹ of the powder of *P. notoginseng* and equal volume of normal saline for the blank group. At 50 min after drug dosing, orbital blood samples were collected into heparinized microfuge tubes and immediately centrifuged at 3000 rpm for 15 min to obtain plasma, which was precisely collected and stored at -20°C until analysis.

In order to reduce complexity of plasma matrix, the extraction was carried out by adding quadruple volume of MeOH to the plasma, and the mixture was vortexed for 30 s and then centrifuging at 12,000 rpm for 10 min to precipitate the denatured proteins. The supernatant was evaporated to dryness under a gentle N₂ stream at room temperature and then re-dissolved with 5 mL TOL-EtOH (7:3, v/v) and collected as analytical samples.

2.6.2. Enrichment of PNS from plasma samples by MISPE

Firstly, 500 mg of SBA-15@MT-MIPs and SBA-15@MT-NIPs were packed manually in an empty solid-phase extraction (SPE) column. Each cartridge was rinsed with MeOH-HAc (9:1, v/v), and no template was detected in the obtained solution by HPLC. After being conditioned with 5 mL TOL-EtOH (7:3, v/v), 5 mL of analytical samples were loaded into the cartridge and sealed it for 12 h. Subsequently, it was washed with 5 mL of washing solution to remove the interfering substances and then was dried thoroughly by a vacuum pump. Finally, it was eluted with 5 mL of MeOH-HAc (9:1, v/v) to get the desired PNS. Both the washing and elution fractions were evaporated to dryness under a gentle N₂ stream at room temperature, and the obtained residues were re-dissolved in 1 mL MeOH for HPLC analysis.

2.6.3. Chromatographic analysis

The amount of PNS was analyzed by an HPLC system. A Shimadzu shim-pack C₁₈ column (250 mm × 4.6 mm, 5 μm) was used as the stationary phase. The mobile phase consisted of ultra water (A) and ACN (B) and was run in an isocratic mode at a flow rate of 1.0 mL min⁻¹. The following gradient was performed: 0–23 min, 23% B; 23–40 min, 35% B. The column temperature was maintained at 35°C. Aliquots of 20 μL were injected. Monitoring and quantitation of PNS were performed at 204 nm.

Agilent 1200 liquid chromatograph combined with an Agilent 6410B Triple Quad mass spectrometer was used to identify PNS in biological samples. An Agilent zorbax eclipse plus C₁₈ column (150 mm × 2.1 mm, 5 μm) was used as the stationary phase. The mobile phase for LC-MS analysis consisted of ACN-H₂O (5:5, v/v) at a flow rate of 0.5 mL min⁻¹.

3. Results

3.1. The optimization of preparation conditions of SBA-15@MT-MIPs

The successful preparation of MIPs largely depends on the judicious selection of the functional precursor, such as functional monomer, solvent/porogen, and their relative molar ratio, etc. So, the preparation conditions of SBA-15@MT-MIPs should be optimized.

3.1.1. The selection of the functional monomers

The selectivity of SBA-15@MT-MIPs is closely related to the number and shape of binding sites within the polymer network structure, as well as the rigidity of the complexes formed by template molecule and functional monomer [36]. Therefore, monomer selection is the first priority for the synthesis of SBA-15@MT-MIPs with good affinity and high selectivity. In this study, three potential functional monomers (4-VP, AM and MAA) were screened to prepare the polymers. In Table 1, SBA-15@MT-MIPs-3 showed the highest *Q* (108.9 μmol g⁻¹) and *IF* values (2.11). It was concluded that the monomer based on AM created SBA-15@MT-MIPs with higher affinity binding sites for PNS compared to other study monomers. This may be explained by the efficient formation of hydrogen bonds between carboxylic groups of AM and templates containing hydroxyl groups. Therefore, AM was chosen as the functional monomer in the experiment.

In addition, the ratio of functional monomer to the cross-linker has an influence on binding capacity and rigid polymer network. In this study, 1:4 was the optimum molar ratio of monomer to cross-linker according to previous paper reports [37,38].

3.1.2. The proportion of template and functional monomer

Generally, the ratio of template to functional monomer has influence on binding affinity and imprinting effect of the polymers, so we examined the effect of different molar ratios of template to functional monomer in a range of 1:3–1:6. The results in Table 1 revealed that the SBA-15@MT-MIPs-3 had the highest adsorption capacity and selectivity with the molar ratios of 1:4, while other molar ratios showed lower adsorption capacity and selectivity.

The reason may be that fewer templates induce less binding sites in polymers due to fewer template-monomer complexes, but residual templates produce lower specific binding capacity and higher non-specific binding capacity [39]. So, 1:4 was selected as the optimum molar ratio of template to functional monomer to construct SBA-15@MT-MIPs.

3.1.3. The optimization of polymerization porogens

The selectivity and affinity of synthesized SBA-15@MT-MIPs through non-covalent interaction between active sites of SBA-15@MT-MIPs and imprinted molecules are closely related to the polarity of the porogens. In addition, porogens have a substantial impact on the self-assembly of template-functional monomer and the size of mesoporous and macropores in the particles. The proper polarity of porogens, the stronger the affinity of SBA-15@MT-MIPs is. Porogenic solvents investigated

in this study were THF, EtOH and EtOH-ACN (2:3, v/v) and these are common organic porogens that are normally used during the synthesis of MIPs [40]. The results were shown in Table 1. When using THF and EtOH-ACN (2:3, v/v) as the porogens, the *Q* and *IF* of the SBA-15@MT-MIPs were not the highest. It was indicated that the hydrogen bond interaction between PNS and AM in EtOH was stronger than the other choices mentioned. So SBA-15@MT-MIPs were prepared in EtOH in this study.

From all the above, the three parameters including the selection of the functional monomers, the molar ratios of template to functional monomer and the optimization of polymerization porogens have been optimized and the optimal material was SBA-15@MT-MIPs-3, which was employed for further study.

Table 1. The preparation of SBA-15@MT-MIPs under different conditions.

Polymer	Functional monomer	Molar ratio ^a	Solvents	<i>Q</i> (μmol g ⁻¹)	<i>IF</i> ^b
SBA-15@MT-MIPs-1	4-VP	1 : 4 : 16	EtOH	75.51	1.74
SBA-15@MT-MIPs-2	MAA	1 : 4 : 16	EtOH	81.30	1.46
SBA-15@MT-MIPs-3	AM	1 : 4 : 16	EtOH	108.9	2.11
SBA-15@MT-MIPs-4	AM	1 : 3 : 12	EtOH	68.71	1.02
SBA-15@MT-MIPs-5	AM	1 : 5 : 20	EtOH	93.00	2.03
SBA-15@MT-MIPs-6	AM	1 : 6 : 24	EtOH	94.50	2.07
SBA-15@MT-MIPs-7	AM	1 : 4 : 16	THF	31.57	1.82
SBA-15@MT-MIPs-8	AM	1 : 4 : 16	EtOH-ACN (2:3)	93.22	1.04

^aThe molar ratio refers to template:functional monomer:cross-linker. ^bImprinting factor (*IF*)=*Q*_{MIP}/*Q*_{NIP}

3.2. Characterization of SBA-15@MT-MIPs

3.2.1. FT-IR analysis

The FT-IR spectra ascertained the successful synthesis and modification of SBA-15. Meanwhile, SBA-15@MT-MIPs had been prepared successfully. As shown in Figure 2A-a, the absorptions at 3464 cm⁻¹ and 1634 cm⁻¹ were ascribed to the stretching and bending vibrations of the O-H, while the peculiar peaks at 1087 cm⁻¹ and 798 cm⁻¹ were related to the characteristic Si-O-Si stretching vibration, indicating that SBA-15 was successfully synthesized. The relatively strong bands in the range of 2800-3000 cm⁻¹ corresponded to the stretching vibration of C-H bonds from the methyl (or methylene) groups of MPS (Figure 2A-b) [41]. The spectrum of SBA-15-MPS (Figure 2A-c) showed new characteristic peak at 1699 cm⁻¹, which was ascribed to the stretching vibration absorption of the C=O in ester groups. The fact suggested that mesoporous carriers were successfully modified by MPS, indicating that the vinyl-terminated SBA-15 was obtained.

The FT-IR spectra of R₁, Rg₁, Rb₁, SBA-15@MT-MIPs and SBA-15@MT-NIPs before eluting templates were explored, and the corresponding results were presented in Figure 2B. The as-prepared SBA-15@MT-MIPs before eluting templates (Fig 2B-d) clearly displayed the characteristic peaks of R₁, Rg₁, and Rb₁, including the peaks at 1078 cm⁻¹ and 1045 cm⁻¹ corresponding to [42], while the peaks were not shown in the spectrum of SBA-15@MT-NIPs (Fig 2B-e). It can be inferred that R₁, Rg₁, and Rb₁ were existed in SBA-15@MT-MIPs before elution. The peak at 1726 cm⁻¹ demonstrated the existence of EGDMA and AM at SBA-15@MT-MIPs and SBA-15@MT-NIPs. In conclusion, the

results indicated that MIP layers were grafted onto the surface of SBA-15-MPS through the free-radical polymerization method.

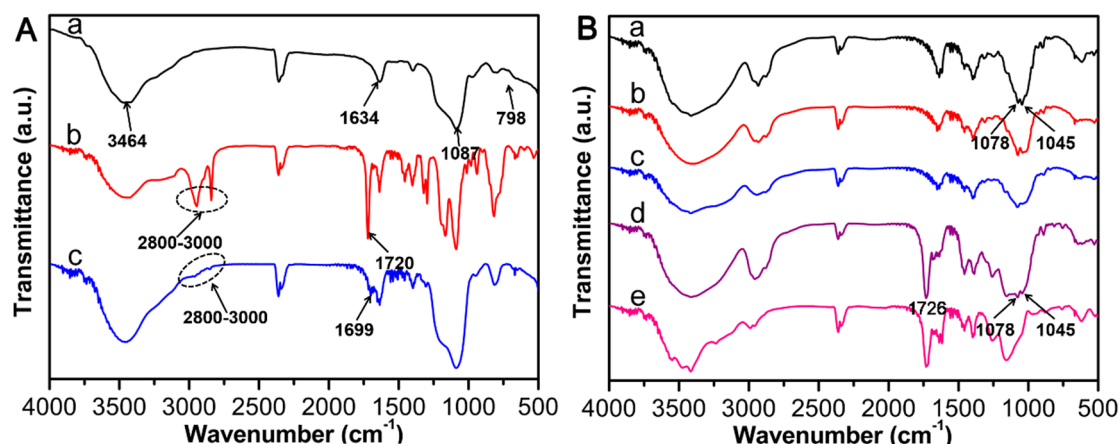


Figure 2. (A) FT-IR spectras of (a) SBA-15, (b) MPS and (c) SBA-15-MPS. (B) FT-IR spectras of (a) R₁, (b) R_{g1} and (c) R_{b1}, (d) SBA-15@MT-MIPs before eluting templates, (e) SBA-15@MT-NIPs.

3.2.2. SEM and TEM analysis

The representative morphology and microstructure of SBA-15 and SBA-15@MT-MIPs were characterized by SEM and TEM, respectively. The SBA-15 with hexagonal rod-like structures was observed in Figure 3A. Compared with SBA-15, significant differences in morphology and particle shape can be seen from Figure 3B, SBA-15@MT-MIPs with a honeycomb-like form exhibited high density of macropores, which were ascribed to the generated imprinted polymer thin layers. Besides, the results confirm that agglomerated particles had nano-sized which provide suitable structure for SPE adsorbents. To investigate the morphology and microstructure thoroughly and intuitively, the TEM images of prepared materials were obtained. As can be seen in Figure 3C and D, all samples displayed the well-ordered hexagonal mesoporous structures, similarly indicating the structure of SBA-15 was maintained after modification and polymerization. However, noticeable differences between them can be distinguished in the images. As illustrated in Figure 3D, the imprinted polymer layers with uniform thickness were densely combined with the surface of mesoporous carriers. As expected, we can posit that the novel MT-MIPs based on mesoporous carriers were successfully prepared. Moreover, the thin imprinted layers could be beneficial for the mass transfer between templates and the surface of SBA-15@MT-MIPs.

3.2.3. TGA analysis

To investigate the thermostability and solid content of the materials, Figure 4 shows the TGA curves of SBA-15, SBA-15-MPS, and SBA-15@MT-MIPs in the temperature range from 25 to 700°C. As shown in Figure 4a, The TGA curve of SBA-15 had only one weight loss stage in the temperature below 100°C, which was assigned to the release of physically and chemically adsorbed water in SBA-15. Figure 4b illustrates that SBA-15-MPS had two weight loss steps in the range of 25-700°C. The first step below 100°C was the release of physically adsorbed water and solvent residues in SBA-15. The second weight loss step showed around 14% weight loss in the temperature range of 100-550°C, which corresponded to the degradation of the MPS silane coupling agent. Analogously, in the TGA curve of SBA-15@MT-MIPs (Figure 4c), the first weight loss step corresponded to the surface adsorbed water. The next much higher weight loss (around 82%) was observed from 100 to 482°C, which could be assigned to the removal of the organic content in the imprinted layers. Meanwhile, it was confirmed that imprinted polymer layers were successfully synthesized.

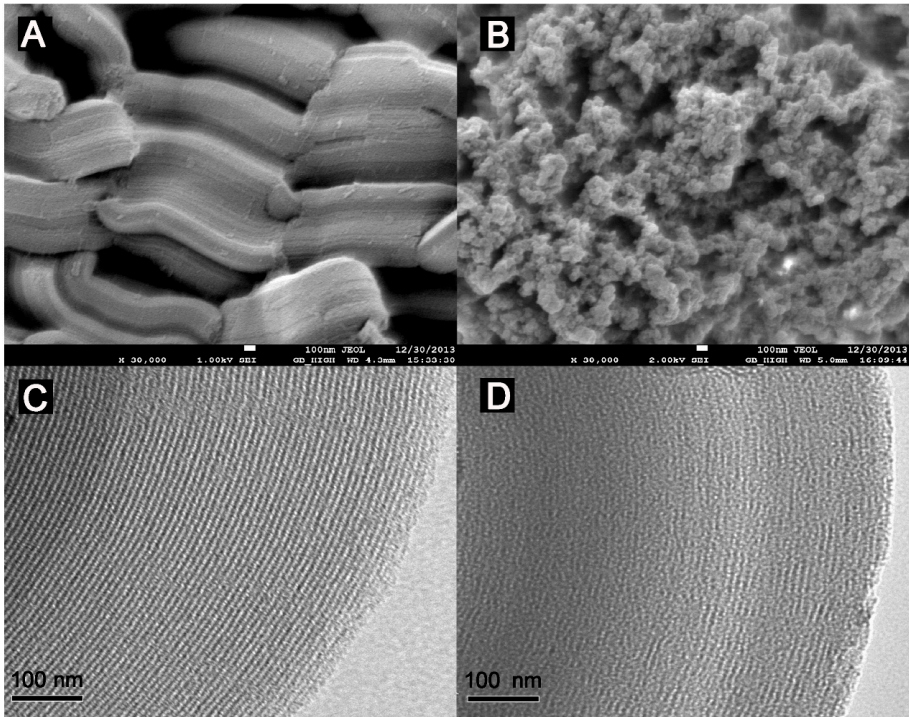


Figure 3. SEM images of (A) SBA-15 and (B) SBA-15@MT-MIPs; TEM images of (C) SBA-15 and (D) SBA-15@MT-MIPs.

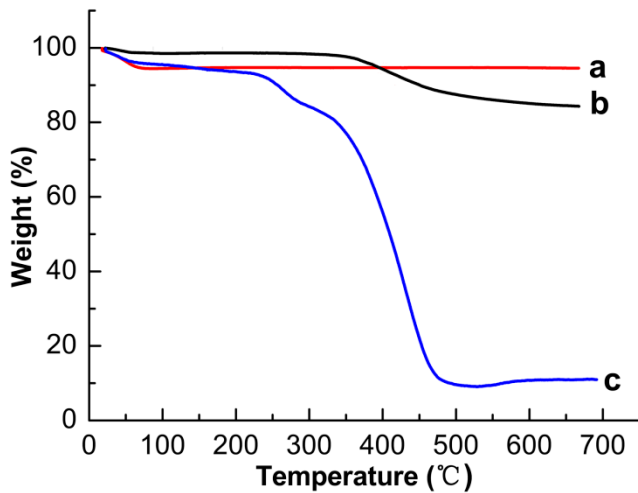


Figure 4. Thermogravimetric weight loss curves of (a) SBA-15, (b) SBA-15-MPS and (c) SBA-15@MT-MIPs.

3.2.4. N₂ adsorption-desorption analysis

N₂ adsorption-desorption analysis was used to evaluate the porosity changes of the mesoporous SBA-15 by the introduction of MPS and MIP layers. The surface areas were measured with Brunauer-Emmett-Teller (BET) theory. Total pore volumes and pore size distributions were obtained based on the adsorption branch of the isotherm with the Barrett-Joyner-Halenda (BJH) method. The results were shown in Table 2. After the attachment of MPS, the BET surface area, pore volume, and pore size of SBA-15-MPS decreased slightly compared with SBA-15. However, the surface area, pore volume, and pore size of SBA-15@MT-MIPs decreased drastically to 140.2 m² g⁻¹, 0.1018 cm³ g⁻¹, and 34.28 Å, respectively. These changes provided a proof that the imprinted layers existed in the nanopores of SBA-15, which has been reported elsewhere [43].

Table 2. Porosities of polymers determined by N₂ adsorption-desorption analysis.

Sample	S _{BET} (m ² g ⁻¹)	V _T (cm ³ g ⁻¹)	D _P (Å)
SBA-15	631.7	1.128	68.79
SBA-15-MPS	436.9	0.7433	64.52
SBA-15@MT-MIPs	140.2	0.1018	34.28

3.3. Investigation on the performance of SBA-15@MT-MIPs

3.3.1. Study of binding properties

The binding properties of polymers towards PNS was investigated by the static adsorption equilibrium experiment and adsorption kinetic studies.

Binding isotherm is a measure of the concentration dependent recognition behavior of a system. As shown in Figure 5A, the adsorption capacities of SBA-15@MT-MIPs and SBA-15@MT-NIPs for three saponins increased rapidly in the beginning stages, and later slowed down with the increasing of initial concentration. Then the adsorption tended to be stable gradually and become constant at 3 mmol L⁻¹, due to the saturation of recognition sites. The saturated adsorption capacities of SBA-15@MT-MIPs and SBA-15@MT-NIPs for PNS were calculated to 123.11, 58.41 μmol g⁻¹, respectively. The IF of the SBA-15@MT-MIPs for PNS was also found satisfactory (IF=2.11). Evidently, the number of PNS bonded on the SBA-15@MT-MIPs cavities was larger than that on the corresponding SBA-15@MT-NIPs at all the concentrations studies. This obvious preferential adsorption was ascribed to the SBA-15@MT-MIPs contained special binding sites complementary in size and shape to the template molecules and the existence of hydrogen bonds between the functional groups and template molecules.

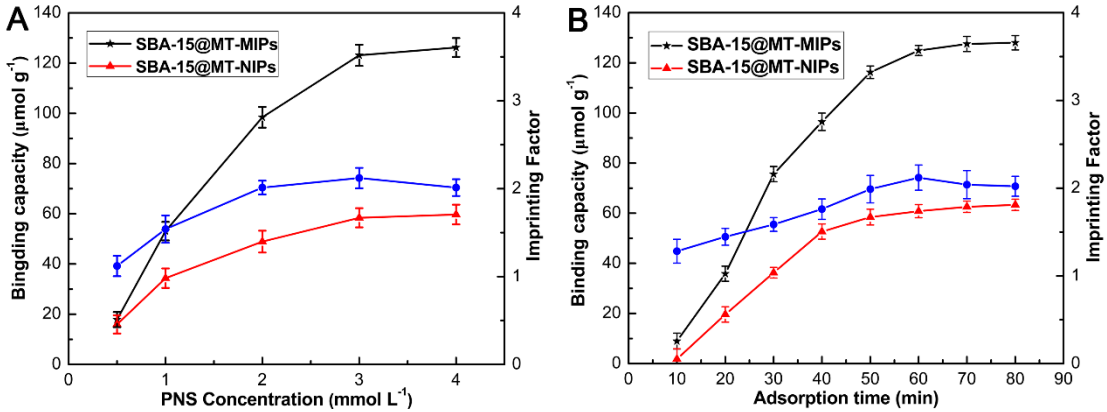


Figure 5. (A) Adsorption isotherms and imprinting factors for SBA-15@MT-MIPs and SBA-15@MT-NIPs with different concentrations of PNS, where IF was defined as $IF = Q_{MIP}/Q_{NIP}$; (B) Adsorption kinetic behaviors of SBA-15@MT-MIPs and SBA-15@MT-NIPs for Rg₁, Rb₁, R₁ with corresponding imprinting factors.

The adsorption kinetic study can provide valuable information about both the binding and the rate-controlling mechanism. As indicated in the Figure 5B, the adsorption rate of the SBA-15@MT-MIPs towards PNS increased sharply in the first 30 min, and then slowed down gradually to reach equilibrium within 60 min. The maximum absorption capacity of SBA-15@MT-MIPs for PNS was 123.11 μmol g⁻¹ and the value of IF was 2.12. In addition, the dynamic curve of SBA-15@MT-NIPs showed a similar trend with that of SBA-15@MT-MIPs but the lower adsorption amount. These could occur because the specific recognition sites of SBA-15@MT-MIPs were generated in the molecular imprinting process and most of the binding sites were located in the proximity of the interior surface.

So target species could easily diffuse into the recognition sites of SBA-15@MT-MIPs, leading to the higher binding capacity and faster mass transfer rate. The results indicated that SBA-15@MT-MIPs with fast sorption kinetic were favorable for the SPE process to separate PNS from plasma samples quickly.

3.3.2. Molecular selectivity of the SBA-15@MT-MIPs

To measure the specificity of SBA-15@MT-MIPs, UA and OA were chosen as the reference compounds. From Figure 6A, we can see that the Q and IF of the SBA-15@MT-MIPs towards the templates of Rb_1 , Rg_1 and R_1 were significantly higher than those of the structural analogs, UA and OA. However, the binding capacities of SBA-15@MT-NIPs for the five analytes were almost the same and all lower than those of SBA-15@MT-MIPs. These results confirmed that SBA-15@MT-MIPs possessed higher selectivity and affinity toward PNS owing to the existence of the specific recognition sites between the monomers and template molecules. As the competitive structural analog UA and OA, although UA and OA are similar to PNS in the molecular volume and structures, the recognition sites were not complementary to the structural analogs. In other words, the memory of specific functional groups also played an important part in conformation memory [44].

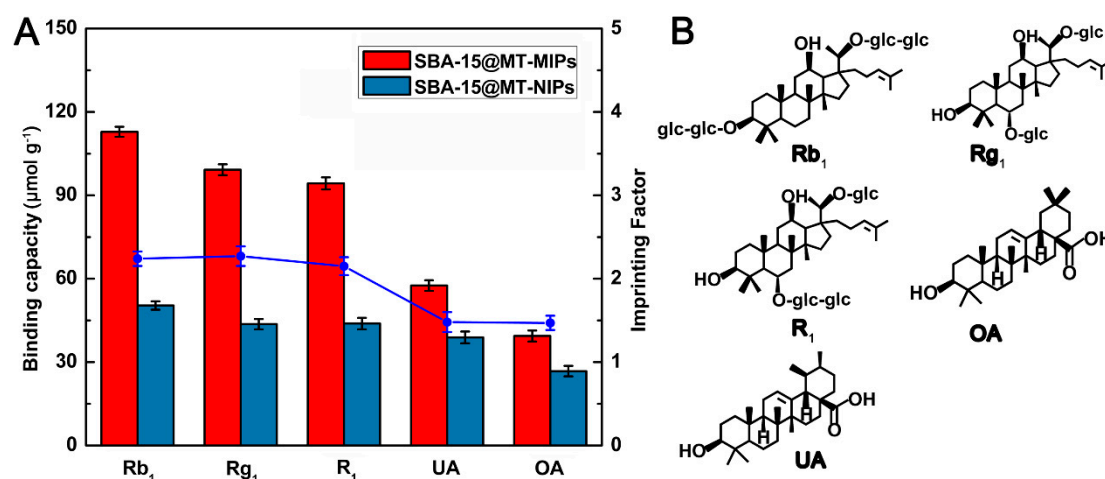


Figure 6. (A) Selective adsorptions of SBA-15@MT-MIPs and SBA-15@MT-NIPs towards Rb_1 , Rg_1 , R_1 , UA and OA standard solution with corresponding imprinting factors; (B) Molecular structures of Rb_1 , Rg_1 , R_1 , UA and OA.

3.3.3. Reusability of SBA-15@MT-MIPs

The stability of SBA-15@MT-MIPs was evaluated by comparing the adsorption capacity towards PNS at each cycle. As shown in Figure 7, the adsorption capacity decreased slowly with an increase in cycle times. After six adsorption-desorption cycles, the adsorption capacity was about 9.09% less. The slight decrease in adsorption capacity mainly resulted from the partial destruction of the recognition sites during the elution procedure. The results indicated that recognition, interaction, and adsorption processes occurred reversibly and the polymers had excellent stability and acceptable reusability. So, it could be used for practical applications.

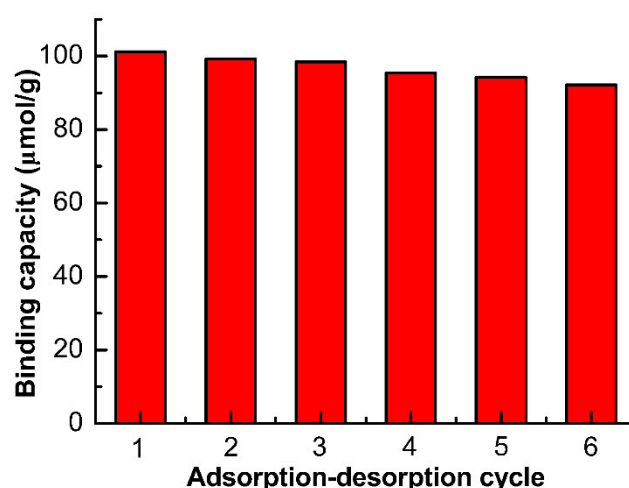


Figure 7. Reusability of SBA-15@MT-MIPs.

3.4. Separation and determination of PNS in real samples

3.4.1. Optimization of the MISPE protocols

The washing procedure is a necessary procedure which could minimize the interferences for the analysis step and activate the binding sites of SBA-15@MT-MIPs for maximizing their interaction with the target analytes. Generally, low-polar solvent was preferred as washing solvent such as chloroform and toluene to disrupt the nonspecific binding between the polymers and matrix components [47]. Initially SPE cartridges containing 500 mg of SBA-15@MT-MIPs and SBA-15@MT-NIPs were loaded with 5 mL of 3.0 mmol L⁻¹ PNS solution in blank rat plasmas and then 5 mL of each washing solvent was applied. For optimizing the condition of the washing step, the various washing solvents, such as H₂O, EtOH, ACN, EtOH-H₂O (5:5, v/v), TOL-EtOH (9:1, v/v) and TOL-EtOH (7:3, v/v) were studied. The results were shown in Figure 8. It can be observed that at least 70% of the PNS were washed off from the SBA-15@MT-NIPs cartridge after it was washed by above five washing solvents, because there was only physical adsorption for SBA-15@MT-NIPs. However, when using TOL-EtOH (7:3, v/v) as washing solvent, the impurities in samples could be mostly cleaned up and the recoveries of PNS were more than 92.47% in MISPE. Following are the reasons. Firstly, TOL-EtOH (7:3, v/v) can induce the formation of hydrogen bonding between PNS and functional monomers, and at the same time interfering compounds were easier washed. Secondly, the specific interaction between the PNS and SBA-15@MT-MIPs was easily disrupted by highly polar solvents (H₂O, EtOH, ACN, EtOH-H₂O (5:5, v/v)). Thus, TOL-EtOH (7:3, v/v) as the optimal washing solution was chosen for running the subsequent experiments.

The selection of eluent plays a key role in excellent sensitivity and precision. It was reported that MeOH-HAc (9:1, v/v) exhibits the good recovery [45,46]. The reason may be that HAc can break the formation of hydrogen bonding between template and functional monomer, and template desorption was easier [47]. Thus we applied 5 mL of MeOH-HAc (9:1, v/v) as eluent agent.

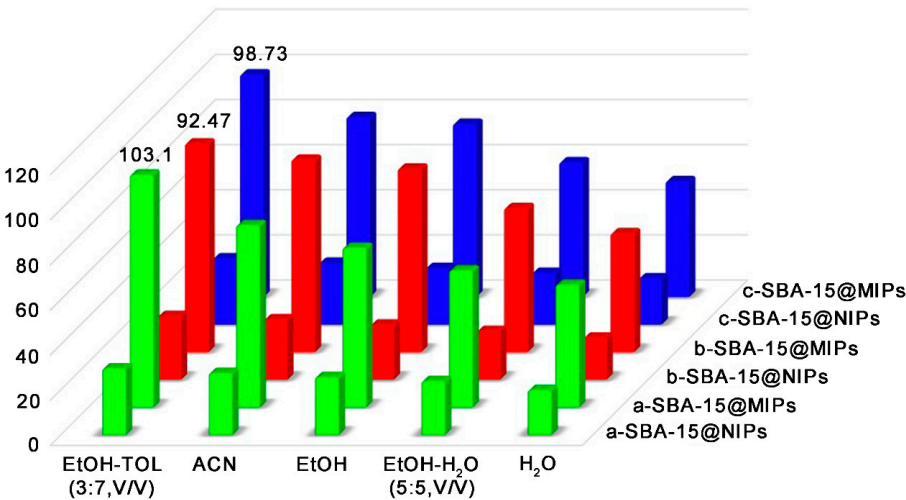


Figure 8. Recoveries of R_1 , R_{g1} , R_{b1} in the washing fractions of SBA-15@MIPs and SBA-15@NIPs with different washing solvents (a: R_1 , b: R_{g1} and c: R_{b1}).

3.4.2. Validation assay

Under optimized conditions of MISPE coupled with HPLC, the linearity, the detection range (LODs) and limit (LOQs) were investigated. The results were shown in Table 3. Calibration curves were established for R_1 , R_{g1} and R_{b1} in rat spiked plasmas over a concentration range of 2.3–47.3 ng mL⁻¹ with benign regression coefficients $R^2 > 0.995$. The LODs and LOQs were determined based on the signal to noise (S/N) ratio of 3 and 10, respectively. The LODs and LOQs of the proposed method for R_1 , R_{g1} and R_{b1} were in the range of 0.63–0.75 ng mL⁻¹ and 2.1–2.5 ng mL⁻¹, respectively. The relative standard deviations (RSDs) for peak areas were calculated to be in the range of 2.2–3.0 for five replicate analyses, which demonstrated that the developed method had a satisfactory reproducibility.

Table 3. The performance parameters of the proposed method (n=5).

Compounds	Linear range (ng mL ⁻¹)	R ²	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)	RSD (%)
R_1	2.3–19.8	0.996	0.63	2.1	2.5
R_{g1}	3.7–47.3	0.995	0.75	2.5	3.0
R_{b1}	3.2–44.5	0.995	0.69	2.3	2.2

The accuracy and practicability of the method were verified by spiked recovery and summarized in Table 4. Since no PNS in the collected the rat plasma samples of was detectable by the proposed method, spiked recoveries were carried out of the samples spiked with 2.5–16 ng mL⁻¹ R_1 , R_{g1} and R_{b1} to evaluate the developed method. As can be seen, the average recoveries at the middle concentration of the developed method for R_1 , R_{g1} and R_{b1} were 101.4%, 93.4% and 94.3%, respectively, with RSD <4.2%. The results indicated a suitable improvement in the recovery and accuracy even at the low concentration was found, and suggested that SBA-15@MT-MIPs can directly be used for selective adsorption and detection of R_1 , R_{g1} and R_{b1} in biological samples.

Table 4. Spiked recoveries of R₁, Rg₁ and Rb₁ in plasma samples (n =3).

Compounds	Concentration taken (ng mL ⁻¹)	Found (ng mL ⁻¹)	Recovery (%)	RSD (%)
R ₁	2.5	2.6	104.3	4.2
	5.0	5.1	101.4	4.0
	10.0	9.9	99.0	3.8
Rg ₁	4.0	3.8	95.0	3.7
	8.0	7.5	93.4	3.6
	16.0	15.2	95.0	3.3
Rb ₁	3.5	3.3	94.3	3.6
	7.0	6.6	94.3	3.3
	14.0	13.5	96.4	3.5

3.4.3. Application to the determination of trace PNS in real samples

To evaluate the enrichment and impurity-removing property of the MISPE column and whether the method is suitable for low concentration detection, the proposed method was applied to the analysis of PNS in rat plasma samples and the results were shown in Figure 9. As shown in Figure 9a, because of extremely low concentration, the analytes were hardly detectable without performing the separation and enrichment of process. Figure 9d shown that most of the analytes and matrix components were washed out from the non-imprinted polymer solid-phase extraction (NISPE) column, and few analytes were detected in the elution solution obtained from NISPE (Figure 9e). However, most of the matrix components only with few analytes were eliminated efficiently after being separated by MISPE column (Figure 9b). From Figure 9c, R₁, Rb₁ and Rg₁ were remarkably concentrated and the interfering peaks arising from complex biological matrices were suppressed, indicating that the remarkable preconcentration ability and desired specific recognition of the novel MISPE sorbents to R₁, Rb₁ and Rg₁. In addition, the amounts of R₁, Rb₁ and Rg₁ that were detected from the rat plasma samples were 2.5, 4.0 and 4.6 ng mL⁻¹, respectively. Finally, the target analytes in the elution solution obtained from MISPE sorbents have been structurally identified based on HPLC-MS and the experimental results were listed in Table 5.

Table 5. The detected chromatographic and spectrometric data of the separated compound in the HPLC chromatograms.

Peak	Compounds	Formula	Retention time (min)	[M+H] ⁺ (m/z)	Reference
1	R ₁	C ₄₇ H ₈₀ O ₁₈	16.88	932.5	[48]
2	Rg ₁	C ₄₂ H ₇₂ O ₁₄	24.34	824.5	[49]
3	Rb ₁	C ₅₄ H ₉₂ O ₂₃	36.34	1109.31	[50]

As could be observed from the chromatograms, the sensitivities of three saponins in plasma samples were greatly enhanced with the MISPE coupled with HPLC-UV analysis. Furthermore, due to the selective binding sites at the surface of SBA-15@MT-MIPs, the MISPE can reach the equilibrium quickly, and this process does not require special instrumentation, consumes much less toxic organic solvent, and has a good clean-up and concentration effect for the analytes. These results demonstrated that the MISPE coupled with HPLC offers a suitable method for selective separation and determination of trace saponins in biological samples.

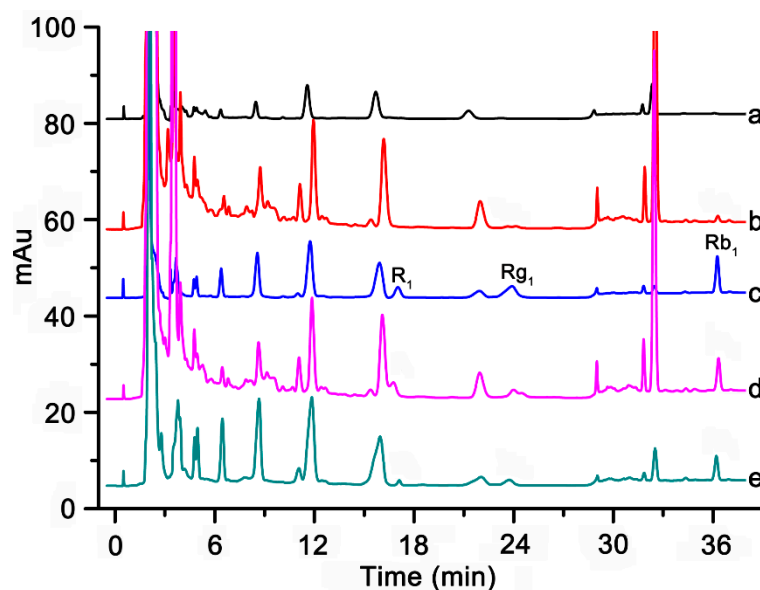


Figure 9. Chromatograms of Rb₁, Rg₁, R₁ in rat plasma samples (a) by direct injection without enrichment, (b) the washing solution protocol obtained from MISPE, (c) the elution solution protocol obtained from MISPE, (d) the washing solution protocol obtained from NISPE, (e) the elution solution protocol obtained from NISPE.

4. Conclusions

In the present study, the innovative multi-template imprinted polymers based on SBA-15 have been prepared by combining the merits of surface imprinting technique and mesoporous material. The obtained SBA-15@MT-MIPs exhibited good characteristics such as excellent adsorption capacity, fast equilibrium kinetics and favorable selectivity towards three templates. Moreover, wide linear range, low LOD and good recovery were obtained, indicating the great applicability for selective separation of specific analytes from complex samples. Finally, the rapid, reliable and simple approach has been successfully employed for the efficient remove and quantitative enrichment of the trace level of PNS from plasma samples simultaneously in a single run. In conclusion, the developed method will find wider applications in diverse clinical and toxicological laboratories for the routine analysis of PNS in biological samples.

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Author Contributions: Shuying Hou and Lina Chen conceived and designed the experiments; Chenghong Sun and Jinhua Wang performed the experiments; Jiaojiao Huang analyzed the data; Dandan Yao and Lei Zhang contributed reagents/materials/analysis tools; Chong-Zhi Wang and Chun-Su Yuan wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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