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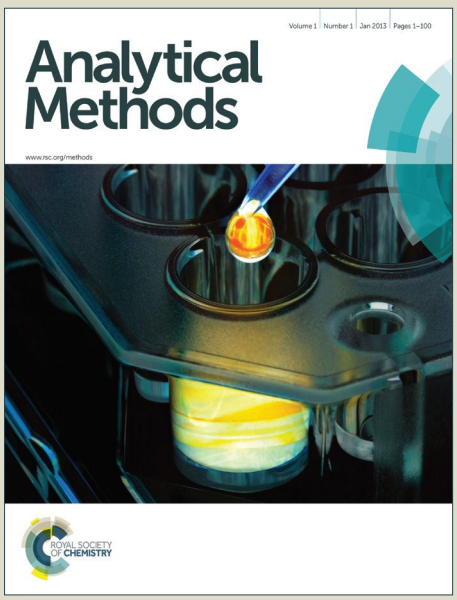

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Green synthesis and evaluation of isoquercitrin imprinted polymers for class-selective separation and purification of flavonol glycosides

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Abstract

A method of solid-phase extraction (SPE) against isoquercitrin (ISO) from natural plant extracts was proposed based on molecularly imprinted polymers (MIPs). The efforts in the present work aim at the emphasis on the topic of "green" chemistry, i.e., the use of green solvent, ionic liquid with a high percentage (63.2%-69.3%) in the total volume of porogenic solvent. For the preparation of ISO-MIPs monolith, 4-vinylpyridine was used as the functional monomer, and ethylene glycol dimethacrylate was the cross-linking monomer, using a mixture of 1-butyl-3-methylimidazoliumtetrafluoroborate (ionic liquid)-N'N-dimethylformamide (DMF)-dimethyl sulfoxide as porogen. It was found that the type of functional monomer, the ratio of template to functional monomer, crosslinking degree, and level of DMF, and the composition of mobile phase greatly affected the retention of the template and performance of molecular recognition. The optimal MIPs were used as solid-phase extraction (SPE) sorbents for purification of ISO, hyperoside, and astragalin and a SPE protocol was optimised for the type of loading solvent, amount of MIPs, washing and elution solvent. It was found that the most suitable solvents for loading, washing and elution step were methanol-water (70:30, v/v), methanol-water (20:80, v/v) and acetonitrile-water (30:70, v/v), respectively. The highest recovery rate of ISO, hyperoside, and astragalin was 87.78%, 93.26% and 83.25%, respectively, from the crude extract of cotton flower.

1. Introduction

Flavonoids are phenolic substances found widely in nature with a range of biological and pharmacological activities.[1-3] Both the flavonoids and their metabolites have display an in vivo antioxidant activity, which is due to their ability to reduce free radical formation and to scavenge free radicals. Therefore, the capacity of flavonoids to act as antioxidants in vitro has been the subject of studies in the past years, and the important structure–activity relationships of the antioxidant activity have been established. Up to date, the antioxidant efficacy in vivo of flavonoids has been less thoroughly documented, possibly due to the limited knowledge on their pharmacokinetics derived from the lack of highly pure flavonoids.

Isoquercitrin (Quercetin-3-O-glucoside) (ISO) and its analogues (Fig. 1), hyperoside, and astragalin are the important flavonoids found in flowers of Gossypium herbaceum L. and leaves of Apocynum lancifolium Rus. and related species. Because of the complexity of the sample matrix, ISO and its analogues need to be separated from interference prior to analysis and a number of analytical techniques have been developed.[4-7] Among them, high-performance liquid chromatography (HPLC) is commonly used[8-12] and pretreatment steps before chromatographic analysis are often required for extracting as well as cleaning up the target analytes from sample. However, to achieve highly pure flavonoids from vascular plants is difficult because they occur in very low amounts in plant with very rich in interfering substances.

Solid-phase extraction (SPE) is one of the most convenient and high performance

technologies for separation of bioactive compounds from plants.[13] It can help minimize the use of organic solvents which are regulated as priority pollutants. A number of sorbents, e.g., C_{18}, spherical silica, alumina, and carbon material, have been used in SPE in previous reports. However, all of the techniques present some disadvantages as the co-extraction of interfering compounds with a similar polarity to the analytes, which hamper in the subsequent determination of the compounds of interest due to absence of selectivity of absorption fillers.

Recently, molecularly imprinted polymers (MIPs) have emerged as powerful sorbent for the selective solid-phase extraction of single compounds or compound classes from complex matrices.[14-16] These highly cross-linked polymers display binding sites in cavities creating the complementary domains of the given molecule with good physicochemical stability, practicality, and predetermination selectivity to the template molecule as well as to the structural analogues. In recent years, attempts have been made to apply MIPs to SPE with special recognition ability to replace conventional sample pretreatment materials. Advantages of the MIPs solid phase extraction (MISPE) are not only in terms of preconcentration and cleaning of samples, but also selective extraction of target analytes, low cost relatively, good mechanical properties and long life, which is particularly important in complex or highly contaminated samples.

Over the past decade there has been an increased emphasis on the topic of "green" chemistry and chemical processes. These efforts aim at the total elimination or at least the minimization of generated waste and the implementation of sustainable

processes. Any attempt at meeting these goals must comprehensively address these principles in the design of a synthetic route or preparation approach. Utilization of nontoxic chemicals, environmentally benign solvents, and renewable materials are some of the key issues that merit important consideration in a green synthetic strategy. For flavonoids, a number of paper related to flavonoids-MIP have been published,[17-21] but volatile solvent, e.g., organic small molecules, had to be used as porogenic solvent, which does not meet the requirements of the green chemistry.

In view of facts above, we intend to propose a greener approach for the synthesis of imprinted polymer for flavonoids. For this purpose, a green and non-volatile solvent, ionic liquid (IL), was used as porogenic solvent to prepare MIP. As a unique, environmentally friendly solvent of low vapor pressure with excellent solvation qualities and chemical/thermal stability, IL has been used in precipitation polymerization to form MIP nano- or micro-particles,[22,23] as well as MIP monolith.[24-26] However, the use of IL to prepare MIP is still limited and the design of IL-based MIP is challenge because of its polar nature affecting the formation of complexes in traditional non-covalent imprinting method. In the present study, ISO was chosen as template, 4-VP as monomer, EDMA as crosslinker, and a ternary, non-volatile solvent mixture, i.e., IL/DMSO/DMF as porogens to prepare MIP for class-selective separation and purification of flavonol glycosides. The effect of polymerization parameters on the selectivity and affinity of the resultant imprinted polymers was investigated. By our knowledge, this is the first report of the preparation of MIP against ISO.

2 Experimental

2.1 Materials

Isoquercitrin (ISO, 98%), hyperoside (HYP, 98%), catechin (C, 98%) and astragalin (AST, 98%) were purchased from Shifeng Biotechnology Co., Ltd. (Shanghai, China). Ethyleneglycol dimethacrylate (EDMA, 98%) and 4-vinylpyridine (4-VP, 98%) were purchased from Sigma (St. Louis, MO, USA). N' N-Dimethylformamide (DMF, 99.6%) and 1-butyl-3-methylimidazoliumtetrafluoroborate ([BMIM]BF_4, AR) were purchased from Jiecheng Chemical Co., Ltd (Shanghai, China). Methyl gallate (MG, 98%) were purchased from Hongsheng Co., Ltd. (Beijing, China). Gallic acid (GA, 98%) was purchased from Guangtuo Chemical Co., Ltd. (Beijing, China). m-Hydroxybenzoic (MHBA, 98%) acid was purchased from Baishun chemical Co., Ltd. (Beijin, China). p-hydroxybenzoic (PHBA, 98%) and azobisisobutyronitrile (AIBN, AR) were purchased from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Dimethyl sulfoxide (DMSO, HPLC grade) was purchased from Tianjin Jiangtian Pharmachem Technology Co., Ltd. (Tianjin, China). Acrylamide (AM, 98%) was purchased from Tianjin Bodi Pharmachem Co., Ltd. (Tianjin, China). Other regents were HPLC grade. The crude extract from cotton flower was obtained from Xinjiang Technical Institute of Physics and Chemistry.

2.2 Instrumentation

The HPLC system K3800 consisting of UV2000/2000D UV/Vis detector, P2000 high-pressure pump, K3800 chromatography workstation (Kai'ao Technology Development Co. Ltd., Beijing, China) was used. The detection was performed at 254 nm with a flow rate of 0.5 mL/min. All of mobile phases were filtered through a 0.22

μm membrane from Millipore before use. Column void volumes were measured by injection of 20 μL of acetone (0.1%, v/v) in the corresponding mobile phase.

The retention factor, *k'*, is calculated by:[24]

$$k' = \frac{(t_R - t_0)}{t_o} \quad (1)$$

where t_R is the retention time of retained peak, t_0 is the retention time of unretained acetone.

Imprinting factor (IF) is calculated by the equation:[24]

$$IF = k'_{MIP} / k'_{NIP} \quad (2)$$

where k'_{MIP} is the retention factor of the template molecule eluted from the imprinted polymer and k'_{NIP} is the retention factor of the template molecule eluted from the non-imprinted polymer.

2.3 Preparation of ISO-imprinted monoliths

Imprinted monoliths were prepared by following process: the pre-polymerization mixture was obtained by mixing ISO, 4-VP, EDMA, AIBN (20 mg), and a mixture of [BMIM]BF_4/DMF/DMSO, as show in Table 1. Then the mixture was sonicated for 20 minutes and injected into stainless steel column (100 mm×4.6 mm). The column was then sealed and submerged in 60□ water bath for 18 h. After completion of the polymerization, the unreacted reagents were rinsed with acetonitrile. Then the template molecules were removed with methanol/acetic acid (9:1). Blank monolith was prepared in same way without imprinted molecules.

2.4 Scanning electron microscopy

Scanning electron microscopy (SEM) was used for the characterization of the

monoliths. Samples were sputter-coated with gold before obtaining images. All scanning electron micrographic images were obtained by using a Shimadzu SS-550 scanning electron microscope, operated at 15 kV and a filament current of 60 mA.

2.5 Mercury porosimetry

Mercury intrusion and extrusion experiments on the monolithic polymer samples were performed over a wide range for pressures starting in vacuum up to 60,000 psi (1 psi = 6.895×10^{-3} MPa) by a poremaster 60 instrument (Quantachrome Instruments, Boyton Beach, FL, USA). Data acquisition was performed in autospeed continuous scanning mode enabling maximum resolution speed in the absence of intrusion or extrusion and maximum resolution and sufficient equilibration time (sampling time) when intrusion or extrusion was occurring rapidly with changing pressure.

2.6 Separation and purification of isoquercitrin from crude extract of cotton flower

The resulting MIPs were pumped out from the stainless steel column and ground and sieved with 71 μm-sieve. Then 0.9 g of uniform granule was packed into a home-made SPE column (200 mm×9 mm) with a small piece of cotton placed at the end of the column. The crude extract (10.09 mg) was resolved by 0.5 ml of methanol aqueous solution (70%, v/v) and loaded on the MISPE column, which was activated with 10 ml methanol before. The sample of the crude extract was washed and eluted, to separate ISO and its analogues from interferences. All significant variables were investigated. As show in Table S1, the optium SPE protocol was made according to the following steps: the column was rinsed by methanol/water (20:80)(5 ml) and

methanol/water (25:75)(5 ml), respectively. Acetonitrile-water mixture (30:70) was percolated through the MIP cartridge to obtain isoquercitrin and structural analogues and segmented collecting by TLC. The target substance was found in the eluted solution with flushing volume of 3-7 ml. The content of ISO and its analogues in purified sample and crude extract were determined by HPLC. As a reference, the solution of crude extract was analyzed directly on a C18 column.

The HPLC system consisting of a quaternary gradient LPG-3400SD pump, a VWD-3100 detector (including flow cell), WPS-3000SL auto sampler, online degasser and reagent rack and four bottles (Thermofisher, USA) was used. Separation was performed on Sun Fire TM C18 (250 mm×4.6 mm, 5 μm) (Waters).

3. Results and discussion

3.1 Preparation of ISO-imprinted monolithic column

3.1.1 Choice of functional monomer

In general, the relatively strong interactions involved between functional monomers and the template often lead to the imprinted polymer with higher affinity. Since methacrylic acid (MAA) is the most commonly employed functional monomer for non-covalent imprinting, the starting point for the optimization was the previously reported MIP of the poly(MAA-co-EDMA)-type targeted toward ISO, but the column pressure was too high when the ratio of template to monomer was 1:4. We also used non-covalent neutral monomer acrylamide (AM) and to prepare MIP as it forms stronger hydrogen-bonds in polar protic solvent than MAA. In present work, it was found that 4-VP other than MAA or AA (IF < 1) was the optimal monomer, which

may be due to potential complexation through hydrogen bonding of the free hydroxyl groups and the nitrogens of the pyrazine cycle (Fig. 1a). HPLC was used to evaluate the performance of ISO-MIP monolith with a mobile phase of methanol/water/acetate acid (90/9/1, v/v/v), in which water was added to reduce non-specific interactions.

3.1.2 Optimization of ratio of template to functional monomer

The influence of template-monomer (T/M) molar ratio on the imprinting factor of the resultant MIP monoliths was studied because the molar relationship between the monomer and template has been found to be important with respect to the number and quality of recognition sites in MIPs.[28] In this work, we varied the molar ratio of template to monomer by setting the ratio of 4-VP to EDMA of 1:5, a ratio of classic funtional monomer to crosslinker as the starting point of the study. As shown in Fig. S1a, the imprinting factors of the resulting MIPs increase with the increased concentration of the monomer. Obviously, the result may be due to more imprinting sites as the increase in the amount of the monomer. However, further increase in the molar ratio of template to monomer led to MIPs monolith with high back pressure and further evaluation was impossible. The best imprinting factor (>2.5) was obtained on P3, which was prepared with a T/M ratio of 1:5.

3.1.3 Optimization of ratio of functional monomer to crosslinker

The effects of cross-linking monomer on the retention factors and imprinting effect of the resulting MIP monoliths were also investigated (Fig. S1b). We have prepared 4-VP/EDMA-polymers having a functional monomer/crosslinker (M:C) ratio of 1: 5, which contained varying ratio of M:C (1:3, 1:4 and 1:5). It was found

that the optimum ratio of 4-VP to EDMA was 1:4 in terms of imprinting factor (>1.7). In general, high levels of cross-linking agent are used, the imprinting sites retain their shape quite well after removal of the templates. However, the result suggested that high levels of crosslinker might lead to the stiffness of the polymer network increased severely, thus decrease the accessibility of the cavities significantly.[29] As a result, a compromise must be found between an inflexible arrangement of the polymer chains to give high selectivity and an appropriate degree of flexibility, which is necessary for good accessibility of the cavities and rapid attainment of binding equilibrium.

3.1.4 Effect of DMF ratio

Previously, a mixture of DMF-DMSO-[BMIM]BF_4 has been used as porogen to synthesize MIP monolith for polar template.[24] [BMIM][BF_4] was found to be the unique porogen to achieve the MIP monolith with desired chromatographic behaviors, which might be attributed to low degree of polymer swelling in ionic liquid.[22,23] At present investigation, it was found that DMF-DMSO-[BMIM]BF_4 also fitted to prepare ISO-MIP monolith. DMSO was used as a solvent to dissolve adequate amount of ISO, and the minimum amount of DMSO was used to minimize the interference of the electrostatic interactions most commonly utilized between the functional monomers and the template. Thus, the effect of the composition of porogenic solvent on the performance of ISO-MIP monolith was studied with by shifting the amount of DMF in the pre-polymerization mixture (Fig. S1c). It was found that when the percentage of DMF was beyond 8.8%, there was no imprinting effect on the resulting MIP monolith at all. With the decrease in the ratio of DMF in the porogenic solvent,

both capacity factor and imprinting factor of the template increased. However, further decrease in the content of DMF leads to peak split of the template on the resultant MIP monolith. In this work, the optimum content of DMF was 2.2% (v/v) in terms of imprinting factor. It should be noted that both DMF and DMSO used in the preparation of MIP monolith are non-volatile solvent with high boiling point.

3.2 Morphological characterization of MIP monolith

The morphology of the ISO-based MIP (P15) was observed by SEM. As shown in Fig. 2a, the MIP monolith shows an agglomerate of microspheres with a cauliflower form that are fused into a continuous structure. In addition, remarkable macropores could be found in the polymer skeleton, allowing low backpressure even at high flow rate. This macroporous structure is related to green solvent [BMIM]BF_4 used for polymerization. Indeed, ionic liquid generally lead to macroporous structure and lower capacity than apolar solvents.[24,25]

The pore size distribution of the optimal MIP monolith P15 was been further studied with mercury intrusion porosimetry (Fig. 2b). The mode pore size of the MIP monolith, i.e., the pore diameter at the maximum of the pore distribution curve, was 1.3 μm, suggesting larger superpores. The result was in agreement with SEM very well.

3.3 Thermodynamic study

The impact of temperature on the retention of ISO was investigated by shifting temperature from 25◦C to 45◦C on the MIP column (P15). With increasing column temperature, it was observed that the retention factors of solutes declined. Data

obtained from the thermodynamic properties of the separation was evaluated by van't Hoff equation:[24]

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln\Phi \quad (3)$$

$$\ln\alpha = -\frac{\Delta\Delta H}{RT} + \frac{\Delta\Delta S}{R} \quad (4)$$

ΔH, ΔS, ΔΔH, and ΔΔS can be obtained from the slopes and intercepts of linear portion of the relative equations (3) and (4).

Over the temperature range in our experiment, the van't Hoff plots were linear for the analogues of ISO on the MIP monolith (P15)(Fig. 3). As shown in Table 2, the absolute values of ΔH for ISO were larger than the analogues. This suggested that ISO has stronger affinity to the recognition sites, and could form a more stable complex than the analogues during their matching in the micro-cavities on the MIP. Moreover, the fact that $|\Delta\Delta H| > T|\Delta\Delta S|$ indicated that the separation between two analogues on this monolithic MIPs was an enthalpy-controlled process.[24,29]

As shown in Table 2, a value of negative enthalpy suggests interactions between the template and the polymer of hydrogen-bonding, ion-pairing, or van der Waals interactions. The negative entropy indicates an increase in the order of the chromatographic system as the solute was bound by the polymer, which is a result of an energetic penalty the freezing of a rotor Gibbs free energy change.[30] The absolute value of TΔS = 3.40-3.68 kcal mol^{-1} (14.2-15.4 kJ mol^{-1}) is ca. three times higher than the value TΔS = 1-1.4 kcal mol^{-1} per rotor calculated for weak complex. Assuming that at 323 K the binding sites have an open conformation and desolvation effects are

minimal, it is possible to conclude that only a small part of the template molecule is embedded into the binding cavity; otherwise, the energetic penalty will be large.

3.4 Separation and enrichment of ISO and its analogues by MISPE.

3.4.1. Selectivity of ISO MIP

To further evaluate the selectivity of ISO-imprinted polymer, the retention of analogues of ISO, hyperoside, astragalin, C, GA, MG, MHBA, PHBA, has been tested by HPLC in same chromatographic conditions above. As shown in Fig. 5, it indicated that the imprinted molecule was much stronger bonding with specific site than other compounds tested. The IF value of hyperoside, astragalin, quercetin, C, GA, MG, MHBA and PHBA, was 2.16, 1.43, 2.02, respectively. The results indicated that the more great extent of similarity to ISO, the more close value of retention factor of analogues to ISO. Furthermore, the retention time of quercetin on the MIP-monolith was much longer than ISO and its analogues, which may be due to the greater nonpolar nature of quercetin in additional to similar groups to ISO. Thus, the MIP monolith has revealed the potential property for separating ISO and its analogues from crude extracts of plants.

3.4.2. Selection of loading solvent

In general, the loading solvent should not rinse the template molecules from MIP in addition to the ability to dissolve ISO. A number of solvents or solvents mixture, such as methanol, acetonitrile, and methanol-water (90:10, 70:30 and 50:50, v/v), were used to perform scounting experiments. Acetone failed to be as loading solvent due to the low solubility of ISO in it. Keeping the amount of ISO (2 mg) and MIPs

(0.9 g) as constant, the volume of different kind of loading solvent was increased from 2 to 6 ml and the percentage of retention in the volume-point of various loading solvent was measured. In our work, methanol-water (70:30, v/v) was chosen as loading solvent (Fig. 5a) since the solution had lower release percentage (< 0.02%) and better solubility than methanol and methanol-water (90:10, v/v).

3.4.3. Screening of washing solvent

The washing step is the most crucial point during SPE protocol since the washing solvent must break non-specific interactions to discard matrix components. In this investigation, 0.45 g of the MIPs was packed into home-made SPE column and 1 mg of the crude extracts was loaded onto the cartridge as described above. Several solvents, different proportions of methanol-water (20:80, 30:70 and 40:60) (v/v), ethyl acetate and acetone, were adopted respectively as possible acceptor solvents to wash the imprinted polymer from 1 to 6 ml. The mixture of methanol-water (20:80 or 40:60, v/v) led to increased ISO binding to the MIP than others (Fig. 5b). In view of the complexity of real sample, the mixture of methanol-water (20:80, v/v) was considered as the optimized washing solvent. It should be noted that the rinsing volume was increased with the increasing of the amount of imprinted polymers used.

3.4.4. Evaluation of elution solvent

In present study, different volume of elution solvent, including acetonitrile and acetonitrile-water mixture (10:90, 30:70, 50:50 and 60:40) (v/v), was explored to choose the optimal elution solvent. As showing in Fig. 5c, the template molecule can not be eluted out by 6 ml of acetonitrile and acetonitrile-water (10:90, v/v) while

50:50 or 60:40 (v/v) of acetonitrile-water led to all the components eluted together from the MIP. For the extract of cotton flower, good extraction efficiency was achieved by using acetonitrile-water (30:70, v/v) since the mixture seriously disturbs the interaction of monomer to template with predominant hydrophobic effects.[31]

3.4.5 Effect of the amount of MIP in SPE

To evaluate the extraction efficiency, the proposed MISPE method was applied to a diluted extract of cotton flower which mainly contains flavonoids. After optimization of the MISPE protocols, methanol-water (70:30, v/v) of 0.5 ml was used as loading solvent, methanol-water (20:80, v/v) of 5 ml and methanol-water (25:75, v/v) of 5 ml was used as washing solvent, respectively, and acetonitrile-water (30:70, v/v) was adopted as elution solvent. Different amounts of MIPs, i.e., 0.45 g, 0.9 g and 1.9 g, were packed into home-made SPE column then for the specific extraction of ISO from the crude extracts and segmented gather the sample by TLC. As expected, an increase in amount of MIP led to an increase in the bound amount of the template and its analogues (Table S2). 0.9-MIPs (0.9 g) was the optimum rather than 0.45-MIPs and 1.9-MIPs in a compromise of the recovery rate of ingredients and extraction time. The huge peaks from the crude extract of cotton flower were eliminated and very little other unwanted peaks were seen in the rest of the chromatograms (Fig. 6). Different from the results with conventional silica-based material as SPE, the retention time of AST is smaller than ISO and HYP. This may be due to the lacking of hydroxyl in 3'-position of aglycone on the molecular structure of

AST. The highest recovery rate of ISO, HYP and AST was 87.93%, 93.00% and 83.25%, respectively (Table 3).

4. Conclusion

The ISO-MIP was successfully achieved with ionic liquid as the composition porogenic solvent. The selectivity of the ISO-MIP against the structure-related flavonoid glycosides was also demonstrated. In addition, the optimization of MISPE procedure, the amount of imprinting polymer, loading step, washing step and elution step, has been explored. Then the SPE based on ISO-MIP was allowed to purify specifically flavonoid glycosides from the extract of cotton flower with the high recoveries for isoquercitrin, hyperoside, and astragalin. As a conclusion, the approach provided a new method for the separation and preconcentration of ISO and its analogs from natural products. Future work will be undertaken to improve the purity of isoquercitrin and its analogues of the MISPE method.

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Legends

Fig. 1. Structures of ISO and its analogues tested. (a) ISO, (b) HYP, (c) AST.

Fig. 2. Morphologies characterization of ISO-imprinted polymer (P15) determined by (a) SEM (b) mercury intrusion porosimetry.

Fig. 3. Van't Hoff plots by plotting ln*k*' vs. 1/T (a) and ln*a* vs. 1/T (b) on imprinted monolith (P15). Mobile phase: methanol/water/acetate acid (90/9/1, v/v/v); detection wave length: 255 nm; flow rate: 0.5 mL/min; injection: 20 μL; temperature: 25-45◦C.

Fig. 4. Selectivity of ISO imprinted monolith (P15). Mobile phase, methanol/water/acetate acid (90/9/1, v/v/v); velocity of flow, 0.5 ml/min; detection wavelength, 255 nm; injection volume, 20 μl; temperature: 30◦C.

Fig. 5 Optimization of the MISPE procedure. Screening of the appropriate (a) loading solvent, (b) washing solvent, (c) elution solvent.

Fig. 6 Chromatograms of the crude extracts before the MISPE column, after the MISPE column and the eluent from MISPE. (a) HYP, (b) ISO, (c) AST. The mobile phase consisted of solvent A (method) and solvent B (3‰ phosphoric acid aqueous solution) and solvent D (acetonitrile) with following gradient: 12% A, 77% B, 11% D, 0→28 min; 12% A, 77→66% B, 11→22% D, 28→60 min; 12% A, 66→65% B, 22→23% D, 60→90 min. Flow rate, 0.5 ml/min; detection wavelength, 255 nm; injection volume, 20 μl; temperature 30◦C.

Table 1 Preparation protocol for MIP monoliths.

Column NO.	ISO (mg)	4-VP (μL)	AM (mg)	MAA (μL)	DMF (mL)	EDMA (μL)	DMSO (mL)	[BMIM]BF_4 (mL)	AIBN (mg)	T (◦C)	IF
1	209.0	192	--	--	0.24	1359	1.2	2.468	20	60	2.085
2	--	192	--	--	0.24	1359	1.2	2.468	20	60	--
3	167.2	192	--	--	0.24	1359	1.2	2.468	20	60	2.347
4	--	192	--	--	0.24	1359	1.2	2.468	20	60	--
5	139.3	192	--	--	0.24	1359	1.2	2.468	20	60	2.277
6	--	192	--	--	0.24	1359	1.2	2.468	20	60	--
7	167.2	192	--	--	0.24	1019	1.2	2.468	20	60	1.623
8	--	192	--	--	0.24	1019	1.2	2.468	20	60	--
9	167.2	192	--	--	0.24	1699	1.2	2.468	20	60	1.327
10	--	192	--	--	0.24	1699	1.2	2.468	20	60	--
11	167.2	--	127.9	--	0.24	1359	1.2	2.468	20	60	0.967
12	--	--	127.9	--	0.24	1359	1.2	2.468	20	60	--
13	167.2	--	--	514	0.24	1359	1.2	2.468	20	60	0.976
14	--	--	--	514	0.24	1359	1.2	2.468	20	60	--
15	167.2	192	--	--	0.12	1359	1.2	2.588	20	60	2.340
16	--	192	--	--	0.12	1359	1.2	2.588	20	60	--
17	167.2	192	--	--	0.48	1359	1.2	2.348	20	60	1.703
18	--	192	--	--	0.48	1359	1.2	2.348	20	60	--
19	167.2	192	--	--	0.06	1359	1.2	2.648	20	60	2.430
20	--	192	--	--	0.06	1359	1.2	2.648	20	60	--
21	167.2	192	--	--	--	1359	1.2	2.708	20	60	N.D.
22	--	--	--	--	--	1359	1.2	2.708	20	60	--

Table 2 The thermodynamic parameters of molecularly imprinted column (P15)

Analytes	ΔH (kJ mol^{-1})	ΔS ($Jmol^{-1}K^{-1}$)	R	ΔΔH ($kJmol^{-1}$)	ΔΔS ($Jmol^{-1}K^{-1}$)	R
ISO	-16.57	-47.67	0.999	--	--	--
methyl gallate	-12.82	-42.39	0.993	-3.75	-5.28	0.881
gallic acid	-12.75	-42.35	0.996	-3.82	-5.32	0.978
catechin	-15.11	-49.84	0.999	-1.46	-2.17	0.935
MHBA	-12.36	-47.56	0.999	-4.21	-0.11	0.980
PHBA	-13.29	-51.36	0.996	-3.28	-3.69	0.921

Table 3 The IF value and retention parameters of ISO and it analogues on P15.

Entry	MIP monolith		NIP monolith		IF	Recovery rate (%)
	Retention time (min)	Retention factor	Retention time (min)	Retention factor		
isoquercitrin	10.866	3.03	5.445	1.02	2.97	61.26
hyperoside	9.805	2.63	5.645	1.09	2.41	74.75
astragalin	5.708	1.11	4.924	0.82	1.35	57.62
quercetin	32.59	10.88	18.60	5.37	2.02	N.D.
catechin	5.306	0.97	5.023	0.86	1.12	N.D.
MA	5.648	1.09	3.99	0.48	2.28	N.D.
GA	5.062	0.88	3.915	0.45	1.94	N.D.
MHBA	3.967	0.47	3.882	0.44	1.07	N.D.
PHBA	3.848	0.43	3.793	0.41	1.05	N.D.

N.D. not determined

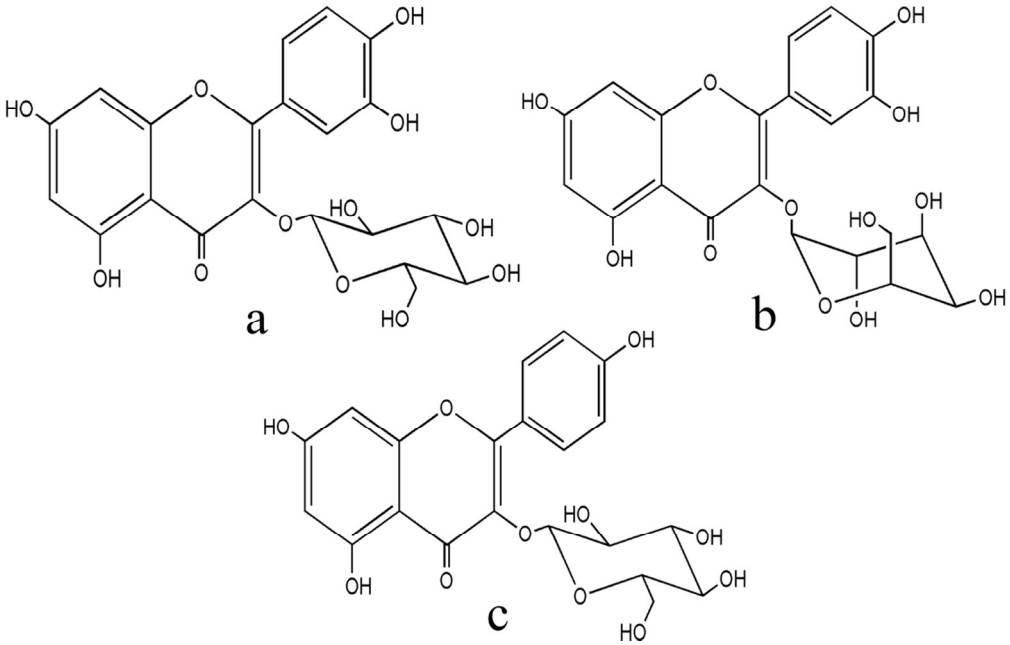

Fig. 1. Structures of ISO and its analogues tested. (a) ISO, (b) HYP, (c) C.
52x33mm (600 x 600 DPI)

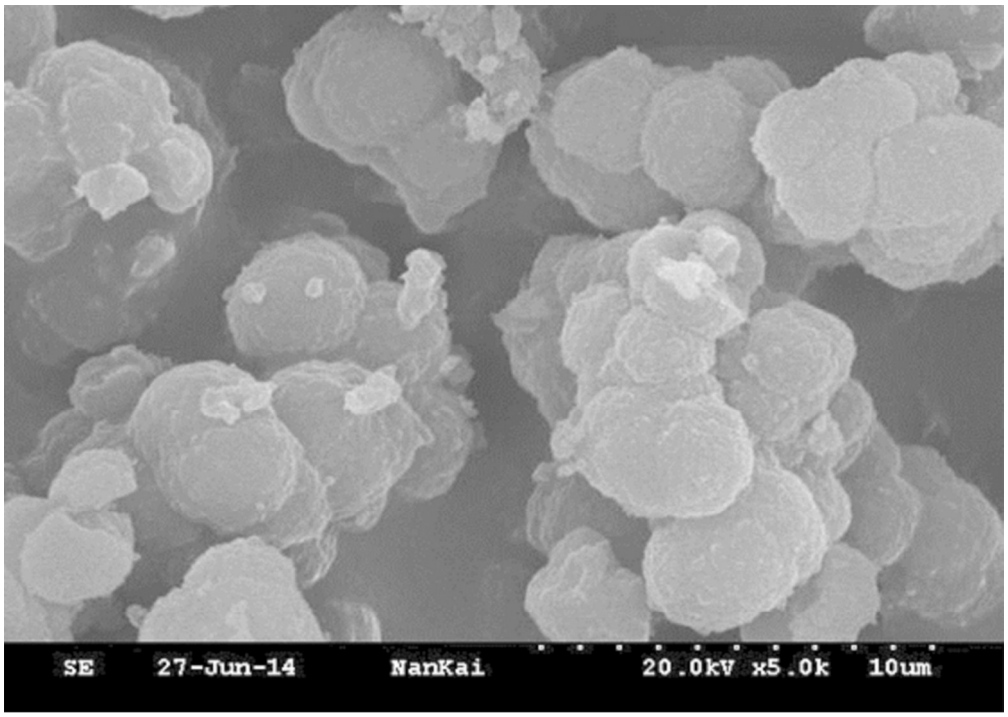

Fig. 2a. Morphologies characterization of ISO-imprinted polymer (P15) determined by SEM.
82x58mm (300 x 300 DPI)

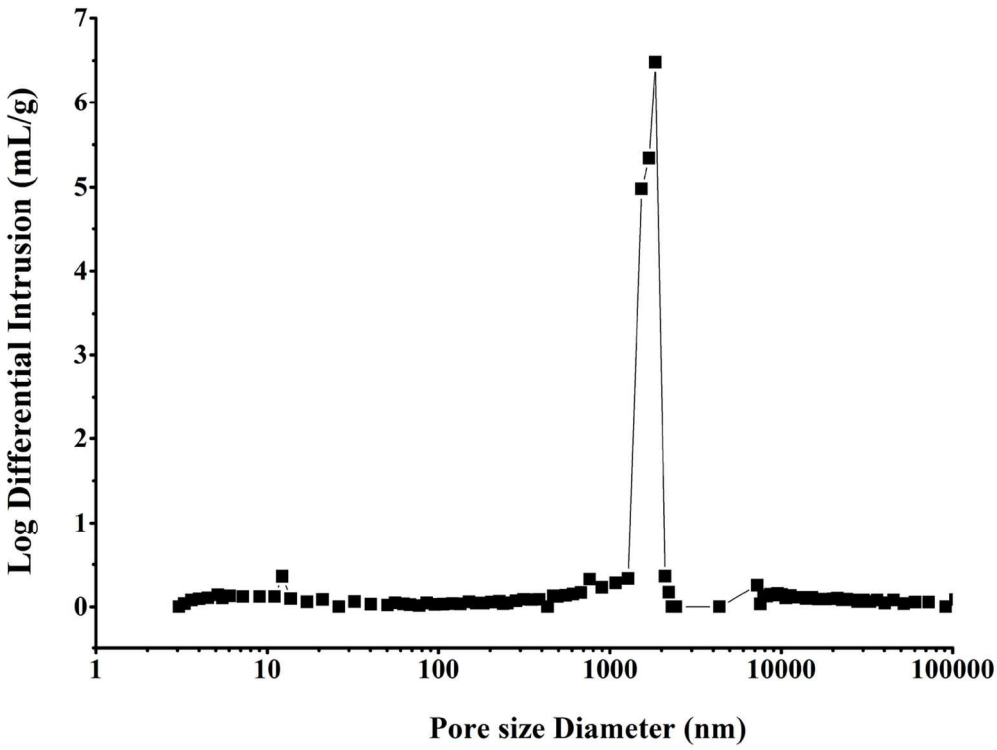

Fig. 2b. Morphologies characterization of ISO-imprinted polymer (P15) determined by mercury intrusion porosimetry.
61x46mm (600 x 600 DPI)

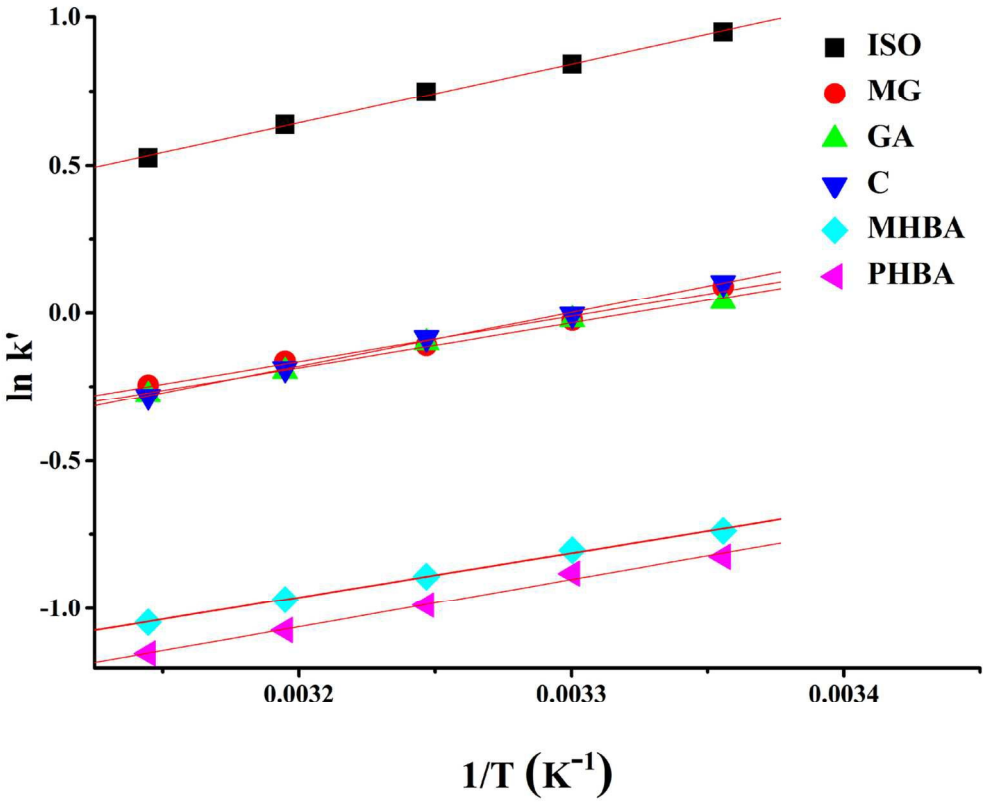

Fig. 3a. Van't Hoff plots by plotting lnk' vs. 1/T (a) on imprinted monolith (P15). Mobile phase: methanol/water/acetate acid (90/9/1, v/v/v); detection wave length: 255 nm; flow rate: 0.5 mL/min; injection: 20 μL; temperature: 25-45◦C.
61x49mm (600 x 600 DPI)

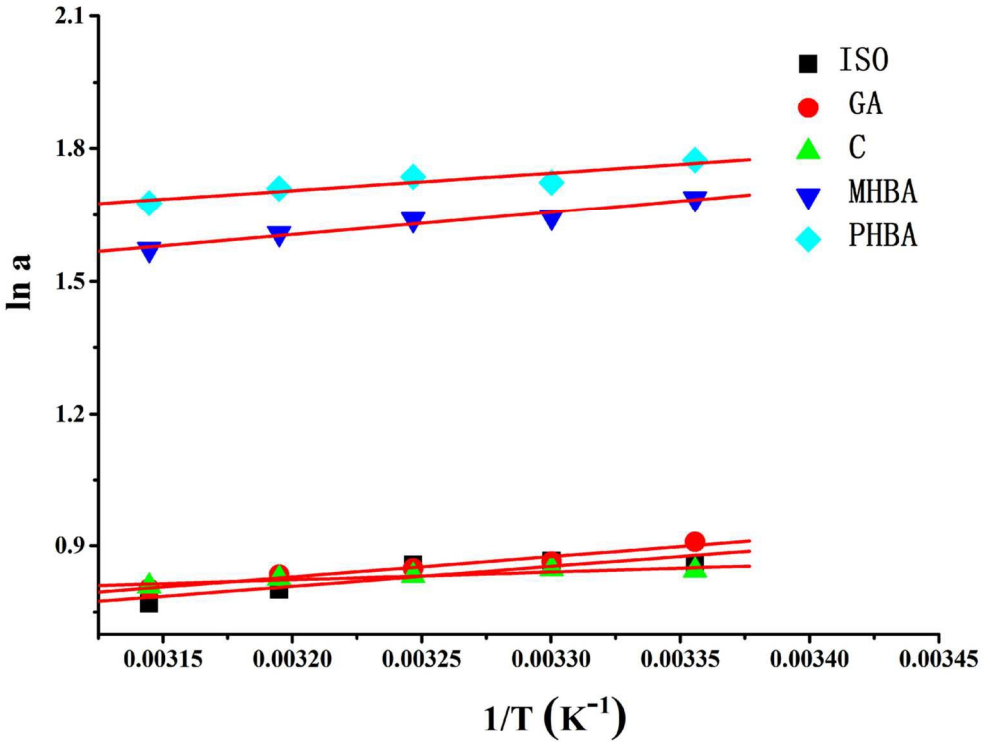

Fig. 3b. Van't Hoff plots by plotting lna vs. 1/T on imprinted monolith (P15). Mobile phase: methanol/water/acetate acid (90/9/1, v/v/v); detection wave length: 255 nm; flow rate: 0.5 mL/min; injection: 20 μL; temperature: 25-45◦C.
56x42mm (600 x 600 DPI)

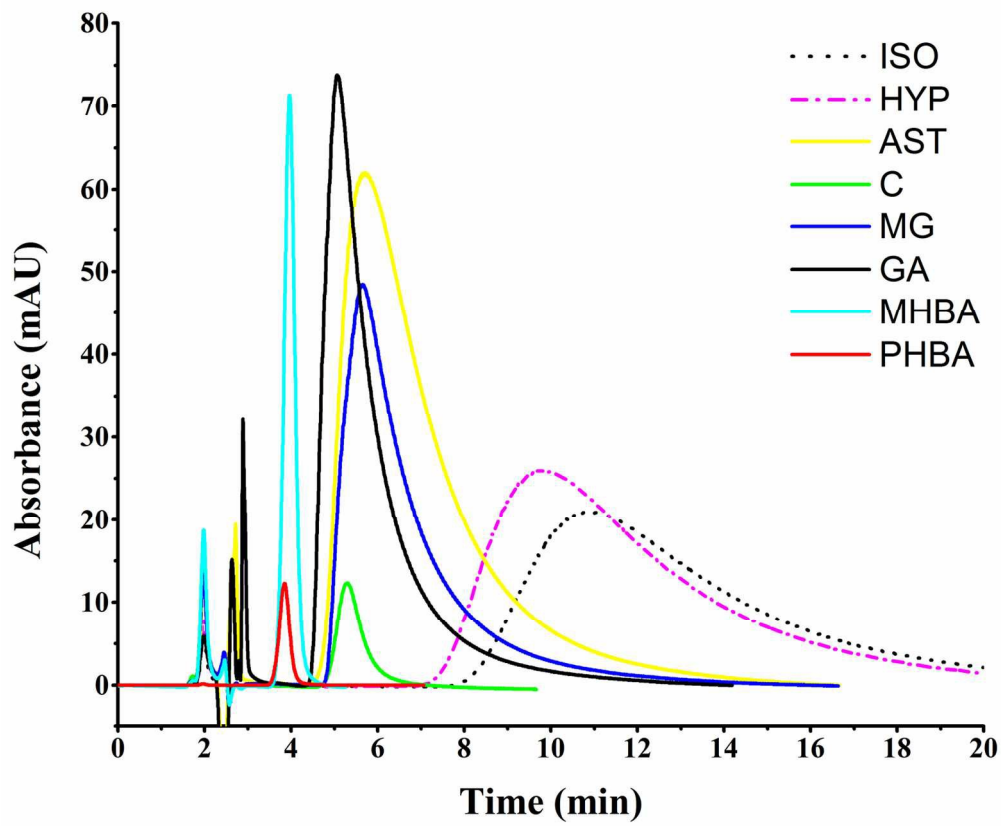

Fig. 4. Selectivity of ISO imprinted monolith (P15). Mobile phase, methanol/water/acetate acid (90/9/1, v/v/v); velocity of flow, 0.5 ml/min; detection wavelength, 255 nm; injection volume, 20 µl; temperature: 30◦C.
69x57mm (600 x 600 DPI)

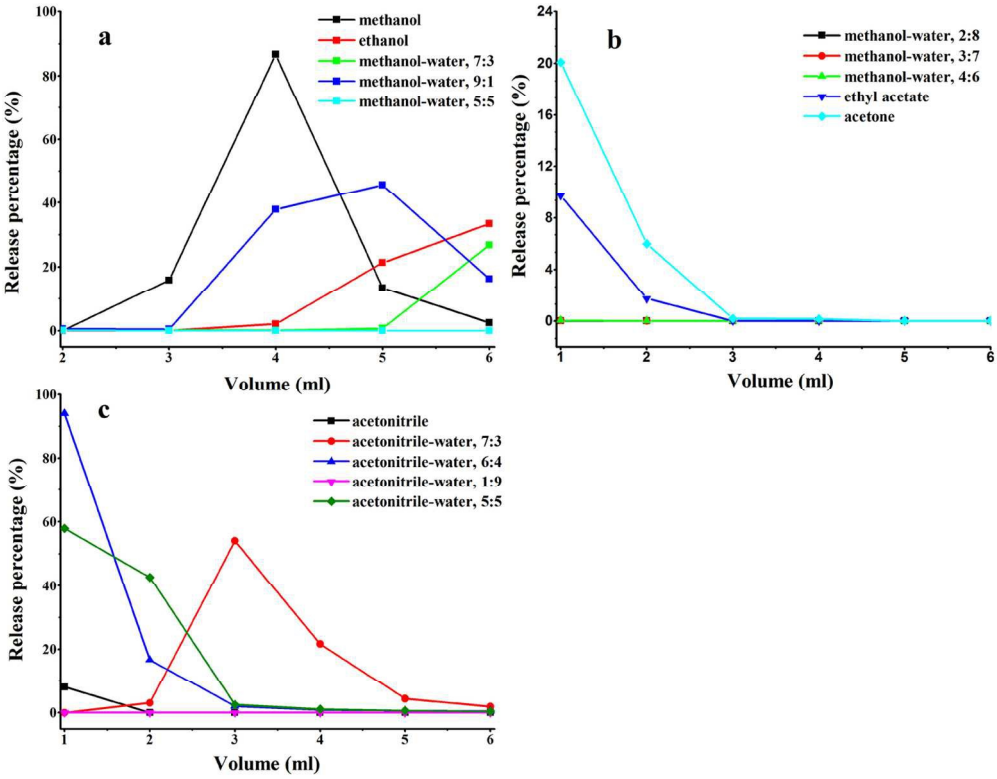

Fig. 5 Optimization of the MISPE procedure. Screening of the appropriate (a) loading solvent, (b) washing solvent, (c) elution solvent.
64x50mm (600 x 600 DPI)

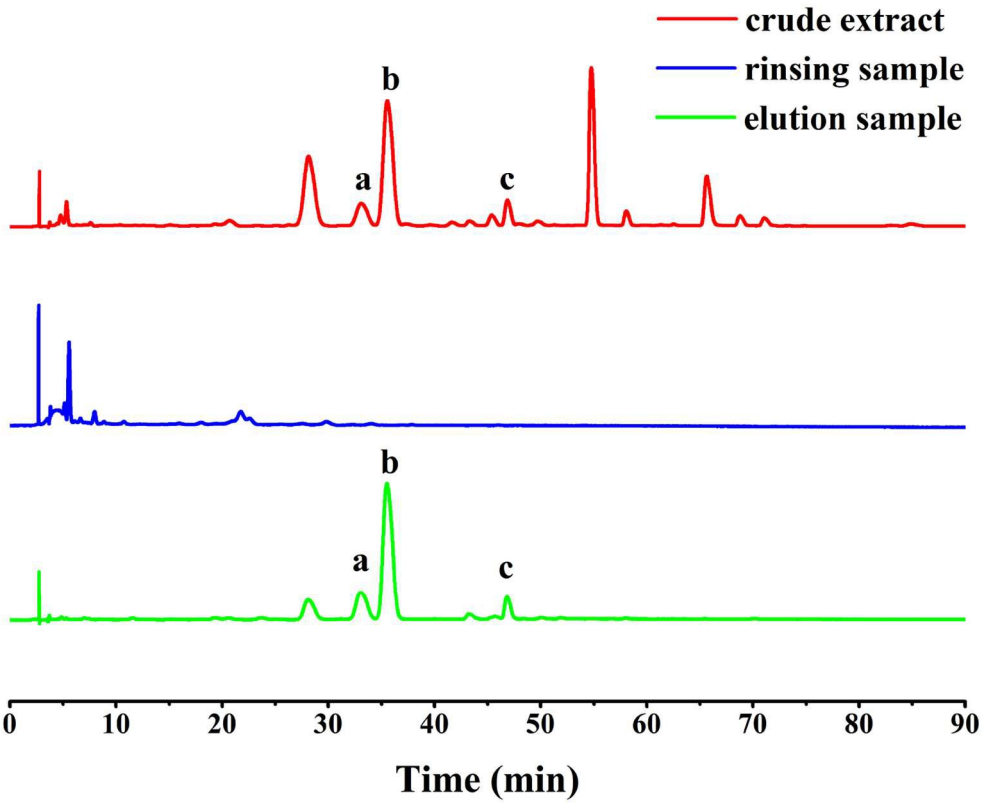

Fig. 6 Chromatograms of the crude extracts before the MISPE column, after the MISPE column and the eluent from MISPE. (a) HYP, (b) ISO, (c) AST. The mobile phase consisted of solvent A (method) and solvent B (3‰ phosphoric acid aqueous solution) and solvent D (acetonitrile) with following gradient: 12% A, 77% B, 11% D, 0→28 min; 12% A, 77→66% B, 11→22% D, 28→60 min; 12% A, 66→65% B, 22→23% D, 60→90 min. Flow rate, 0.5 ml/min; detection wavelength, 255 nm; injection volume, 20 μl; temperature 30◦C.
69x58mm (600 x 600 DPI)