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SEPARATION OF HINOKIFLAVONE FROM *SELAGINELLA DOEDERLEINII* HIERON BY IONIC LIQUID-MODIFIED SILICA GEL

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Abstract

Supported ionic liquids (SILs) have been efficiently used to adsorb and separate a wide range of natural products. In this work, three IL-modified silicas were synthesized including SiO_2•Im^+•Cl^-, SiO_2•Im^+•BF_4^- and SiO_2•Im^+•PF_6^-, which were characterized via infrared spectroscopy, thermogravimetric analysis, inductively coupled plasma analysis, elemental analysis, etc. which were used as adsorbents to separate hinokiflavone (HIN) from *Selaginella doederleinii*. After screening the adsorption effects of different IL-modified silica and influence factors, the optimal adsorption conditions on HIN were as follows: SiO_2•Im^+•Cl^- as adsorbent, adsorption time 30 min, adsorption temperature 30ºC, solid-liquid ratio 1 : 10 g/ml, sample concentration 0.2 mg/ml. Moreover, through the verification of dynamic adsorption and desorption, the results showed that SiO_2•Im^+•Cl^- had strong separation effect on HIN, which can explain that the adsorption method was feasible and provide reference value for the study of adsorption and separation of active components in traditional Chinese medicine.

Introduction

Selaginella doederleinii Hieron, a pteridophyte medicinal herb belonging to the *Selaginellaceae*, is widely distributed in southern China (Jiang *et al.* 2018). Its whole plant has been used to treat cancer, cardiovascular disease and inflammation (Wang *et al.* 2015). Hinokiflavone (HIN) (Fig. 1), known as a biflavonoid, is the main active ingredients in *S. doederleinii* (Li *et al.* 2017) and has a wide range of pharmacological activity, such as antioxidant, anti-inflammatory, anticancer, anti-aging and antiviral effects (Sui *et al.* 2016).

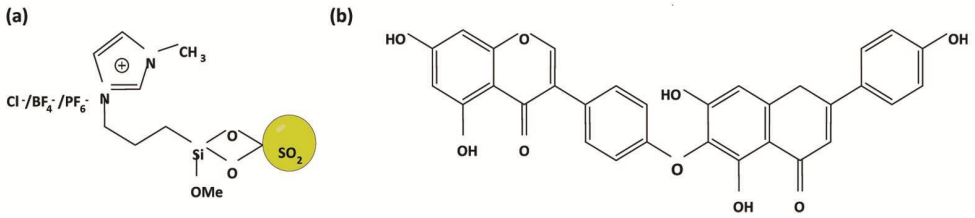

Fig. 1. Chemical structure of ionic liquid-modified silica (a) and hinokiflavone (b).

Ionic liquids (ILs) as green solvents are composed of organic cations and inorganic anions (Zhang *et al.* 2017, Zec *et al.* 2018), which possess the advantages of high thermal stability, low vapor pressure, low flammability and good solubility (Qian *et al.* 2016, Berthod *et al.* 2018). In recent years, IL has been used in the extraction and analysis of natural products (Seitkalieva *et al.* 2018). Supported ionic liquids (SILs) consist of N-alkyl-3-methylimidazolum ILs and adsorption material such as silica gel, which become more and more attention on account of designable structure and high selectivity (Nie *et al.* 2015, Sheikhian and Bina 2016), and has been applied for

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the separation of natural medicines. Tian *et al.* 2009 reported absorbed and separated tanshinone from *Salvia miltiorrhiza* using synthesized SILs as adsorbent. The results showed the effect of absorption and separation on tanshinone was significant. Wang *et al.* 2017 reported improved yield of artemisinin by 25% using SILs without causing its degradation.

Based on the good biological activity of HIN, in this research, after three kinds of different supported ILs were synthesized, they were characterized by infrared spectroscopy, thermogravimetric analysis, inductively coupled plasma analysis, elemental analysis, etc. In addition, a simple, effective and convenient method was developed on the selective adsorption and separation of HIN with SILs. Meanwhile, the adsorption and separation conditions were optimized to improve the yield of HIN from *S. doederleinii*.

Materials and Methods

Selaginella. doederleinii was collected from Phoenix Mountain on 13/09/2018 in Zunyi, China. HIN standard (HPLC ≥ 98 %) was purchased from Ruifensi Biotechnology Co. Ltd., China. N-methylimidazole, sodium tetrafluoroborate, potassium hexafluorophosphate and 3-chloropropyl -3-methoxy-silane were purchased from Aladdin Industrial Corporation. HPLC methanol and acetonitrile were purchased from Shuangjv Chemical Reagent Factory. All other analytical grade agents were purchased from Shanghai Reagent Factory, China.

N-methylimidazole (4.926 g, 60 mmol) dissolved with acetone (100 ml) mixed fully with 3-chloropropyl-3-methoxy-silane (11.923 g, 60 mmol). Then the reactants were refluxed at 100ºC for 12 hrs under the protection of nitrogen. The reactants were filtered and dried under vacuum, which obtained successfully SiO_2•Im^+•Cl^- with a yellow liquid. The above product and $NaBF_4$ (1.097 g, 10 mmol) or KPF_6 (1.841 g, 10 mmol) were dissolved with acetonitrile in a round bottom flask. The solution was refluxed further at room temperature for 12 hrs. Then the reaction solution was filtered and dried under vacuum. Ultimately, SiO_2•Im^+•BF_4^- and SiO_2•Im^+•PF_6^- were triumphantly obtained respectively (Fig. 2).

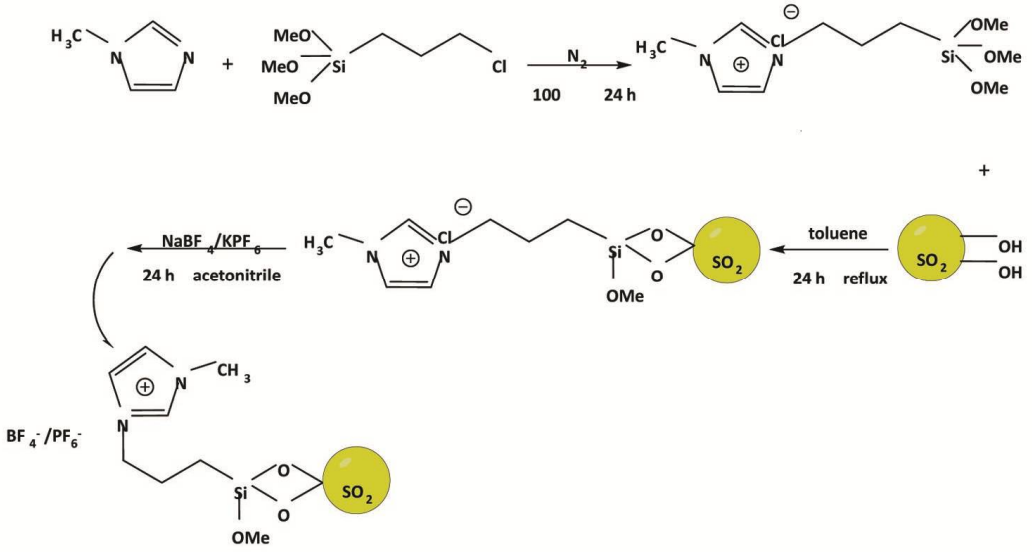

Fig. 2. The preparation process of three ionic liquid-modified silicas.

According to the reports of Wagner *et al.* (2014) and Hu *et al.* (2016), the chemical structures of three SILs were identified by six analysis methods, *i.e.* infrared analysis, thermogravimetry, elemental analysis, inductively coupled plasma, particle size distribution and N_2 adsorption-desorption isotherms.

According to the method of Ji *et al.* (2018), the extract of *S. doederleinii* was prepared under the optimized process for further high performance liquid chromatography (HPLC) analysis. The HPLC analysis of HIN was performed by an Agilent 1260 chromatograph (Santa Clara, USA) equipped with an ACE C18 reversed-phase column (5 µm, 250 × 4.6 mm, Phenomenex, USA). Mobile phase was A (acetonitrile) and B (0.1% formic acid solution) using gradient elution (0 - 10 min, 40 - 50 % (A); 10 - 20 min, 50 - 60 % (A); 20 - 30 min, 60 - 70 % (A); 30 - 40 min, 70 - 80 % (A); 40 - 50 min, 80 - 90 % (A); 50 - 60 min, 90 - 100 % (A)). Other chromatographic parameters were 1.0 ml/min flow rate, 10 µl injection volume, 25°C column temperature and 330 nm measurement wavelength. The calibration curves for HIN was y = 22116 x -108 (R^2 = 0.9995).

To evaluate the adsorb effect on HIN, SILs (SiO_2•Im^+•PF_6^-, SiO_2•Im^+•BF_4^-, SiO_2•Im^+•Cl^-), extraction temperatures (10, 20, 30, 40, 50 and 60ºC), extraction time (10, 20, 30, 40, 50 and 60 min), solid to liquid ratio (1 : 6, 1 : 8, 1 : 10, 1 : 12, 1 : 14 and 1 : 16 g/ml) and sample concentration (0.10, 0.15, 0.20, 0.25 and 0.30 mg/ml) were investigated as single factor variables.

The preparative separation of HIN was performed on a wet packed column (200 mm × 10 mm) with 500 mg of the SILs. Before separation, the extract (100 mg) was mounted on the column and the bed volume (BV) was 10 ml. The extract was eluted to obtain finally the target product under 0.06 MPa for 8 hrs (Fig. 3). Based on the initial results, a stepwise elution method was chosen to separate HIN from *S. doederleinii* by HPLC analysis. In this study, the volume of each collected elution fraction was 4 ml and the flow rate was 0.3 ml/min.

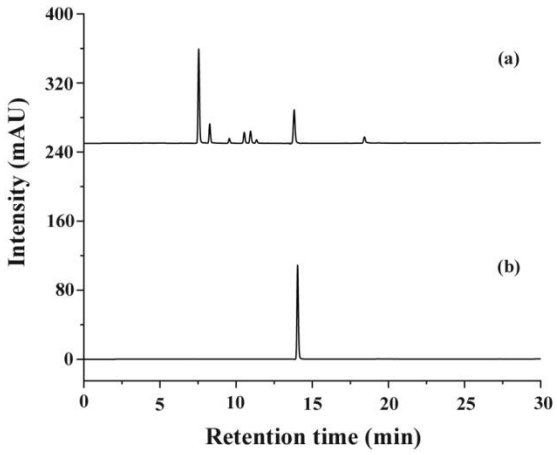

Fig. 3. The HPLC chromatographic profile of *Selaginella doederleinii* (a) and hinokiflavone standard (b).

Results and Discussion

The FT-IR spectra of three synthetic IL-modified silica are presented in Fig. 4 to find out the characteristic absorption peaks between 400 and 4000/cm. The absorption peak of stretching vibrations on O–H bonded silanol groups was 3416/cm within full wave. The peaks at 1046/cm and 799/cm mainly belong to Si–O–Si asymmetric and symmetric vibration, respectively. The absorption peaks within 2900 - 3100/cm were attributable to C–H stretching vibration. In addition,

the peak at 1576 and 1472/cm could be the skeleton stretching vibration of imidazole ring. These results were similar to more or less the data of Nie *et al.* (2015) which could prove that ILs had been successfully bonded on the surface of silica.

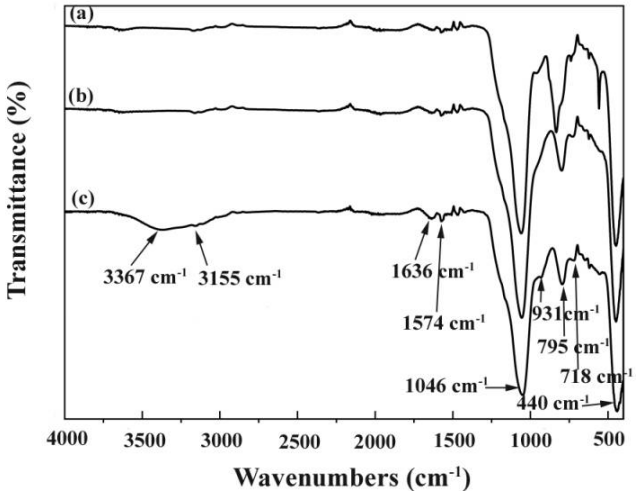

Fig. 4. FT-IR spectra of SiO_2•Im^+•Cl^- (a) SiO_2•Im^+•BF_4^- (b) and SiO_2•Im^+•PF_6^- (c).

Thermogravimetric analysis (TGA) was used to investigate the thermal stability of target products. The SiO_2•Im^+•Cl^- exhibited initially a weight loss at temperature below 250°C which might be due to the removal of few solvent residues (Fig. 5). In the range of 250 - 800°C, the IL-modified silica showed obviously two thermal curves. Firstly, weight loss was within 250 - 400ºC because of decomposition of ILs bonded on the surface of silica. And the second mass loss was within 400 - 800ºC range on account of silanol group's decomposition on the surface. Thus, SiO_2•Im^+•Cl^- showed stability to separate target molecules.

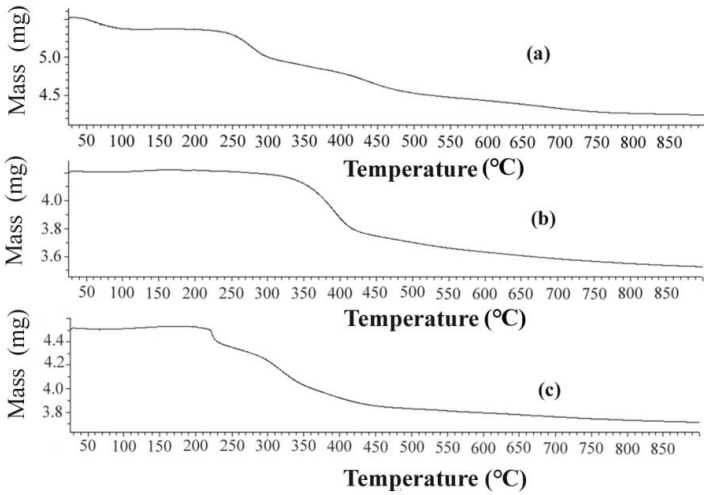

Fig. 5. TGA curves of SiO_2•Im^+•Cl^- (a) SiO_2•Im^+•BF_4^- (b) and SiO_2•Im^+•PF_6^- (c).

Inductively coupled plasma (ICP) can detect non-metallic elements such as carbon, boron, phosphorus, arsenic, chlorine. So, the three IL-modified silicas were determined by ICP. The results showed that chlorine amount of SiO_2•Im^+•Cl^- was 12.1%, boron amount of SiO_2•Im^+•BF_4^- was 3.1% and phosphorus amount of SiO_2•Im^+•PF_6^- was 8.7%, which were basically consistent with the theoretical bonding amount of 12.6, 3.2 and 8.8%, respectively. It indicated that the IL-modified silicas had been successfully synthesized.

The amount of IL-modified silica gel was determined by PE-240C elemental analyzer (German Elemental Analysis Systems, German). The elemental amount of the SiO_2•Im^+•Cl^- was 10.201% of C, 2.335% of H and 4.032% of N, respectively (Table 1). Moreover, N amount of blank sample (silica) was 0 % and the bonding amount of SiO_2•Im^+•Cl^- was calculated to be 2.82 μmol/m^2. In addition, the bonding amount of other IL-modified silica was in the range of 1.53 - 1.61 μmol/m^2.

Table1. Elemental analysis of SILs.

SILs	H (%)	C (%)	N (%)	bonding amount (μmol/m^2)
SiO_2·Im^+·Cl^-	2.335	10.201	4.032	2.82
SiO_2·Im^+·BF_4^-	1.306	7.712	2.446	1.61
SiO_2·Im^+·PF_6^-	1.343	6.951	2.385	1.53

Based on N_2 adsorption - desorption experiment, the pore volume and average pore diameter of SiO_2•Im^+•Cl^- were 0.629 cm^{-3}/g and 6.235 nm, respectively. The particle size distribution of SILs was also a remarkable property in the separation and enrichment of natural medicines with laser scattering. The achieved values of D_{10}, D_{50} and D_{90} on SiO_2•Im^+•Cl^- were 10.137, 22.671 and 41.564 μm, respectively and the average particle diameter was 23.945 μm.

Compared to activated silica, the effects of three different SILs on the adsorption rate of HIN were evaluated. Fig. 6 showed that all IL-modified silica possessed stronger adsorption ability than activated silica, which indicated ILs play an important role in adsorption. The reason might be that hydrophobicity of IL anion could influence the adsorption efficiency of HIN(Liu *et al.* 2014). If hydrophilia of the anions was strong, the adsorption regions could be easily occupied by water in the solution and then the adsorption efficiency would be decreased. Thus, the order of sorption performance for HIN was $Cl^{-1} > BF_4^{-1} > PF_6^{-1}$. Based on the above results, SiO_2•Im^+•Cl^- was the best IL-modified silica and was finally selected for further studies (Fig. 6).

The effect of adsorption time on the adsorption efficiency of the biflavonoid by SiO_2•Im^+•Cl^- was investigated in the range of 10 - 60 min. The adsorption efficiency of HIN was found to be increase from 57.72 to 88.81% with improvement of extraction time as shown in Fig 7a. However, when the extraction time was in the range of 30 - 60 min, the adsorption efficiency had little change.

Through changing the adsorption temperature of HIN, their adsorption effect was investigated in detail. When extraction temperature was raised from 0 to 30ºC, it exhibited that HIN adsorption capacity was obviously increased as shown in Fig. 7b. However, the adsorption efficiency slightly decreased with the rising temperature because of thermally unstable property of target molecules. So, 30ºC was chosen as the optimal extraction temperature.

After screening different IL-modified silica，adsorption temperature and adsorption time, and solid-to-liquid ratio were investigated in detail. As shown in Fig. 7(c), it could be found the sorption capacity also increased when the solid–liquid ratio was changed from 1:16 to 1 : 10 g/ml. In order to save excess solvent, the results indicated HIN was fully adsorbed as the solid-to-liquid ratio increased to 1 : 10 (g/ml).

The initial concentration of HIN was also a key factor on the adsorption of the biflavonoid. Fig. 7(d) showed that the adsorption performance would increase with the enhancement of HIN concentration because of the hydrogen bonding interaction. But, as HIN concentration exceeded 0.2 mg/ml, the adsorption capacity could decrease, which would weaken the interaction between target molecules and IL bond adsorbent. So, 0.2 mg/ml was selected as the optimal HIN concentration.

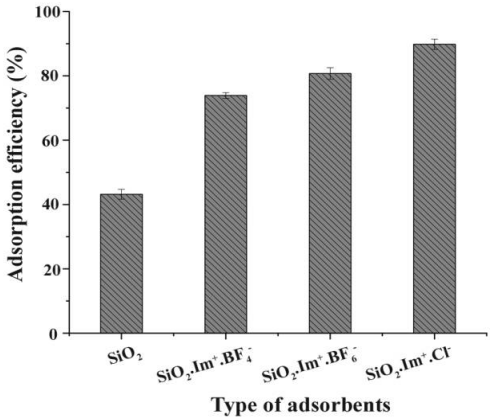

Fig. 6. Effect of different adsorbents on the adsorption efficiency.

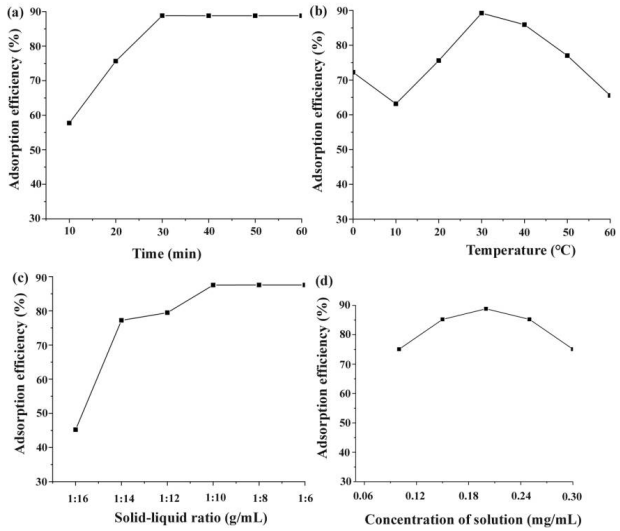

Fig. 7. Effects of different parameters on the adsorption efficiency of hinokiflavone (a) time, (b) temperature, (c) ratio of solid to liquid, (d) sample concentration.

Elution mode and condition is very critical for the separation of HIN from the extract. After screening for different elution solvents, including methanol, ethanol, acetonitrile, phosphoric acid, acetic acid, formic acid, petroleum ether and ethyl acetate, the results suggested that dichloromethane - methanol - 0.01 mol/l formic acid solution (30 : 1 : 0 →8 : 1 : 0.1) possessed the best desorption performance with the stepwise elution program, which finally obtained 2.17 mg of HIN (HPLC > 90%). The whole eluting process was completed within 360 min, and the preparative amount of target constituent could be easily enlarged on the basis of the change of chromatographic column size.

In this study, the adsorption efficiency of three IL-modified silica gel as adsorbents for HIN was evaluated. Firstly, three IL-modified adsorbents were successfully synthesis and identified by the analysis approaches described above. And then the effects of different influence factors, namely solid–liquid ratio, adsorption time, sample concentration and adsorption temperature were all optimized. Furthermore, the preparative separations of HIN were studied in detail using a wet packed column of SiO_2•Im^+•Cl^-. In general, IL-modified silica was suitable for separation and purification of the biflavonoids from *S. doederleinii*, which could provide a valuable approach for the adsorption and separation of the flavonoids from natural products using column chromatography mode.

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