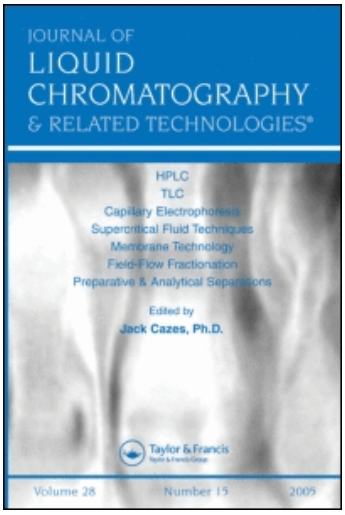


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# Rapid Purification of Huperzine A and B with Two Polystyrene based Resins by Preparative Low-Pressure Liquid Chromatography

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**Abstract:** A rapid, facile, and environmentally friendly process was developed for purification of huperzine A (HupA) and huperzine B (HupB) from the Chinese traditional herb *Huperzia serrata*. The process consisted of two steps of preparative low-pressure liquid chromatography (LPLC) on two polystyrene based resins, successively. The first step removed a large amount of impurities and captured HupA and HupB using Amberlite XAD-4 from the herbal extraction prepared by 1% aqueous $H_2SO_4$. This step was more efficient than multi-cycle liquid-liquid extraction as an initial separation step. The second step was able to separate HupA and HupB, employing a novel uniform polystyrene based porous microsphere named PST as the packing material. The PST column demonstrated a better separation and shorter run time compared with a commercial $C_{18}$ column. The mobile phases used in both LPLC's simply consisted of water and ethanol that are considered to be of low toxicity. Combination of the XAD-4 and PST chromatography and one step of

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crystallization enabled purification of HupA and HupB from 0.18% and 0.08% to 98.2% and 98.8%, respectively, with total recoveries of 82.8% and 84.3%.

**Keywords:** *Huperzia serrata*, Huperzine A, Huperzine B, Preparative low-pressure liquid chromatography, Purification



## INTRODUCTION

Huperzine A (HupA) and huperzine B (HupB) (Fig. 1) are two lycopodium alkaloids extracted from the Chinese traditional herb, *Huperzia serrata*.[1] As reported by Ma et al.,[2] these two alkaloids co-exist in the same herbs of *Huperzia* and related genera, i.e., in most cases. They both are promising drugs for treatment of Alzheimer's disease or myasthenia gravis, for the reversible and selective acetylcholinesterase (AChE) inhibiting activity with relative lack of toxicity.[1,3,4] HupA has been approved in China, recently, as a drug for palliative therapy of Alzheimer's disease, and is also used in the USA as a supplementary drug for correction of memory impairment.[5] HupB has a higher therapeutic index due to its longer duration of action than HupA, although it demonstrates 1/10 of anti-AChE activity in comparison with HupA.[4,6]

Separation of HupA and HupB was very difficult, due to their structural similarity and low contents in natural resources. Current processes for purification of HupA and HupB from *H. serrata* include multiple steps of liquid-liquid extraction (LLE), silica gel column chromatography, and crystallization.[7–9] However, a few limitations and drawbacks of these methods can be observed.

Firstly, the duration of the purification was about 1 month, so the whole process was time-consuming. Secondly, batch-to-batch reproducibility and the recovery might be unsatisfactory, due to the dissolution of the silica gel and the irreversible adsorption of the compounds onto the silica support. Finally, the organic solvents such as chloroform large-scale used in the process were highly toxic and environmentally unfriendly. Therefore, it is necessary to develop a fast and efficient process for purification of HupA and HupB.

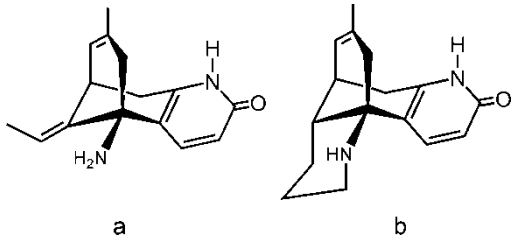


***Figure 1.*** The structures of huperzine A (a) and huperzine B (b).

The adsorption separation method may be preferable to initial multi-steps of LLE because of its simple process and low cost. Compared with classical adsorbents, such as silica gels and activated carbons, polymeric adsorbents are more attractive alternatives for their favorable characteristics such as high mechanical strength, chemical stability, and repetetive use.[10] The most commonly used polystyrene based polymeric adsorbents, such as the XAD family, however, are yield low resolution chromatographic separations because of their relatively large particle sizes (250–600 μm);[11] therefore, they are more suitable for pre-separation of natural products prior to final chromatographic separation.

In the final separation stage, high performance liquid chromatography (HPLC) is often employed to achieve high resolution.[12] However, HPLC is operated under high pressure, using packings with particle sizes less than 20 μm. Therefore, it is considered to be a rather expensive technique, and not really applicable on a large preparative scale.[13] In our previous work, a novel porous spherical styrene-divinylbenzene polymeric resin, named PST, with a 30 μm average particle size, was prepared by combining a microporous glass membrane emulsification technique and suspension polymerization process.[14] Due to its rather narrow size distribution and high porosity, the PST column was successfully used for preparation of icariin[15] and paclitaxel[16] from their crude extracts at low operating pressure, respectively, and demonstrated good selectivity, excellent resolution, and high speed of separation, similar to HPLC.

In this paper, we report a method for preparative purification of HupA and HupB from the herb of *H. serrata* in LPLC mode to ensure an economical process. Regarding the trace amounts of desired products in the herbal extraction, a polymeric adsorbent, Amberlite XAD-4, was employed for the capture of HupA and HupB. The result of this initial adsorption separation was compared with that of LLE. In order to achieve high resolution and high recovery without the use of HPLC, comparative studies were carried out between the PST and commercially available silica-based $C_{18}$ packings. The preparative PST column LPLC process was then developed for subsequent purification of HupA and HupB from the crude extract obtained from XAD-4 LPLC. Ethanol-water was used as mobile phase throughout the two LPLC processes. After crystallization, the highly purified HupA and HupB were characterized by IR, MS, and NMR.

## EXPERIMENTAL

### Materials and Reagents

HupA and HupB were purchased from Hunan Kinglong Bio-Resources Products Industry Co. (Hunan, China) with claimed purities >99%. The herb of *Huperzia serrata* was collected from Changsha, Hunan province of

China. HPLC grade methanol was obtained from Tianjin Shield Company (Tianjin, China). HPLC grade water was obtained from a Rios ultra-pure water system (Millipore, Bedford, USA). All other chemicals were of analytical grade and used without further purification.

The commercial spherical resin, Amberlite XAD-4, was purchased from the Beijing Branch of Rohm Haas Co. (Beijing, China). PST was newly developed by us as reported elsewhere[14–16] and supplied for the work. Table 1 shows the physical characteristics of XAD-4 and PST. The silica-based $C_{18}$ packing, named YWG C18 (10–40 μm), was purchased from Tianjin No. 2 Chemical Plant (Tianjin, China).

## Apparatus



All preparative LPLC runs were carried out on an ÄKTA purifier 100 chromatography system (GE Healthcare, Upplasa, Sweden), comprising a model P-900 pump, a model UV-900 UV-monitor, a model Frac-900 fraction collector, and UNICORNTM software system control. The rotary vacuum evaporator was obtained from Kelong Company (Beijing, China). The apparatus used for HPLC was an Agilent 1100 equipped with an autosampler, a binary pump, a thermostatted column compartment, and a diode-array detector (Agilent Technologies, Palo Alto, CA, USA). Chromatograms were collected using an HPLC 3D Chemstation (Agilent Technologies). The HPLC Inertsil Ph-3 column (250 mm × 4.6 mm ID, 5 μm) was obtained from GL Sciences Inc. (Tokyo, Japan).

The FTIR instrument was a Jasco FT/IR-660 Plus (Jasco, Tokyo, Japan). ESI-MS was performed on a Finnigan LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The $^{1}H$ and $^{13}C$ NMR spectra were recorded using Bruker NMR AV400 and AV600 (Bruker BioSpin GmbH, Rheinstetten/Karlsruhe, Germany), respectively.

An XK 16/20 column (200 mm × 16 mm ID) was obtained from GE Healthcare (Uppsala, Sweden.) The glass column (250 mm × 25 mm ID), used in XAD LPLC, was purchased from Beijing Chemical Reagents Company (Beijing, China).

***Table 1.*** Physical characteristics of XAD-4[a] and PST[b]

| Property: | Particle diameter (μm) | Surface area ($m^2/g$) | Porosity (mL/mL) | Average pore diameter (nm) |
|---|---|---|---|---|
| XAD-4 | 490–690 | ≥750 | ≥0.50 | 5.84 |
| PST | 30 | 745 | 0.54 | 14.7 |

[a]Rohm Haas Technical Bulletin.
[b]Detailed test methods are available in Ref.[15]

## Preparation of the Solvent Extract of *H. serrata*

Dry herbs of *H. serrata* were ground and sieved to about 40 mesh. A batch of 100 g of the herb powder was loaded into a 2 L flask and soaked with 1% (v/v) aqueous $H_2SO_4$, twice, for 24 h at ambient temperature at the solid–liquid ratio of 1:10 (w/v) and 1:5 (w/v), respectively. The extract solution was filtered with a Buchner funnel. The pH value of the combined filtrate (Sample A) was adjusted to 9.0 with 5 M NaOH. The resulting solution was centrifuged at 5000 rpm for 30 min and then filtered through a 0.45 μm cellulose membrane. The filtrate (Sample B) was used as the feed solution for XAD-4 LPLC. The amounts and weight fractions (i.e., purities) of HupA and HupB in the solvent extract were analyzed by HPLC.

## Liquid–Liquid Extraction (LLE)

Initial separation using the LLE method was investigated here to compare with the chromatographic separation using XAD-4. The separation was carried out as described by Liu et al.[7] with some modification. Briefly, 1.5 L of Sample A was extracted with 1.0 L of ethyl ether, and then the aqueous layer was neutralized to pH 7.0 with 5 M NaOH and concentrated to 100 mL with a rotary vacuum evaporator at about 60°C. The resulting solution was further alkalized to pH 9.0 with 5 M NaOH and extracted with 100 mL of chloroform three times. Finally, the chloroform layers were combined and evaporated under reduced pressure with a rotary vacuum evaporator at about 40°C. The residue was re-suspended in methanol for HPLC assay.

## Column Chromatography Separation

### XAD-4 Adsorption Separation

Prior to use, XAD-4 was suspended in 95% (v/v) ethanol-water overnight, and then slurry packed in a 200 mm × 25 mm ID column and equilibrated with 600 mL water at flow-rate 5 mL/min. 1.5 L of Sample B was loaded onto the column. At the end of adsorption, the column was washed with water to remove non-adsorbed impurities, and then washed with 20% ethanol. Finally, elution accompanied with regeneration steps was performed with 95% ethanol. The flow rate of adsorption, washing, elution, and regeneration was maintained at 10 mL/min, and the eluent was detected at UV 311 nm. The collected fraction corresponding to HupA and HupB was evaporated under reduced pressure with a rotary vacuum evaporator at about 60°C. The residue was used as the crude extract for further purification using $C_{18}$ and PST reversed-phase chromatography.

### $C_{18}$ Reversed-Phase Chromatography

A 100 mm × 16 mm ID column was slurry packed with YWG C18 medium and washed with sufficient amount of ethanol. After washing, the column was equilibrated with 20% ethanol. 0.5 g of crude extract was dissolved in 5 mL of 20% ethanol and loaded onto the column. Then, the column was washed with 20% ethanol and eluted isocratically with 30% ethanol. Finally, the column was regenerated with 100% ethanol. The flow rate was 2 mL/min and the detection wavelength was 311 nm. The fractions corresponding to HupA and HupB, respectively, were filtered using a 0.45 μm disposable syringe PVDF filter for HPLC assay.

### PST Reversed-Phase Chromatography

A 100 mm × 16 mm ID column was slurry packed with PST medium, washed with ethanol, and then equilibrated with 30% ethanol. A 0.5 g of crude extract was dissolved in 5 mL of 30% ethanol and loaded onto the column. Then, the column was washed with 30% ethanol and eluted isocratically with 40% ethanol and regenerated with 100% ethanol. The flow rate was 12 mL/min and the detection wavelength was 311 nm. The fractions corresponding to HupA and HupB, respectively, were filtered and analyzed by HPLC.



## Crystallization of HupA and HupB

The collected fractions of HupA and HupB from the PST reversed-phase chromatography were, respectively, evaporated under reduced pressure with a rotary vacuum evaporator at about 60°C until a few crystals appeared. The condensed fractions were put into a refrigerator at 4°C overnight. The crystals of HupA and HupB were collected and dried, and then submitted to MS, IR, and NMR structure determination.

## Analytical Method

### HPLC Analysis

HPLC was used for quantitative analysis of HupA and HupB in the collected fractions from the purification procedures. An Inertsil Ph-3 column was used in this assay. The isocratic mobile phase was methanol-water (90:10, v/v). The flow rate was 1.0 mL/min at 25°C. Assay samples were dried and redissolved in methanol. A 20 μL sample was injected each time and effluent was monitored at 311 nm. HupA and HupB were quantified by comparing the average peak areas of the sample with those of their standards, respectively.

Structure Determination

The collected fractions from PST reversed phase chromatography corresponding to HupA and HupB were directly injected into the mass spectrometer. A spray voltage of 4.5 kv was employed and the temperature of heated transfer capillary was set to 275°C. The mass spectrometer was scanned from $m/z = 100$ to 600 in positive ion mode.

The $^{1}H$ and $^{13}C$ NMR spectra of the crystals of HupA and HupB were recorded at 20°C in $CDCl_3$ using Bruker NMR AV400 and AV600, respectively.

## RESULTS AND DISCUSSION

### Preparation of the Solvent Extract of *H. serrata*

The effects of different extraction conditions have been investigated and reported in our previous studies.[17,18] The relatively efficient and simple extraction method was adopted in this study. Averaging the results (Sample A) of ten batches of 100 g of the dry herb powder, the amounts of HupA and HupB were 20 mg and 9.0 mg, the concentrations were 13.3 mg/L and 6.0 mg/L, and the purities on the basis of dried solvent extract were 0.18% and 0.08%, respectively. The relative standard deviations (RSD) of the amounts and purities for these repeated extractions were less than 5%. As the comparatively low contents of HupA and HupB in the solvent extract Sample A, minor changes in the amounts and the purities of HupA and HupB occurred during further alkalization, centrifugation, and filtration procedures of seven batches to obtain Sample B for adsorption separation. Figure 2 shows a typical HPLC analysis of the solvent extract (Sample B).

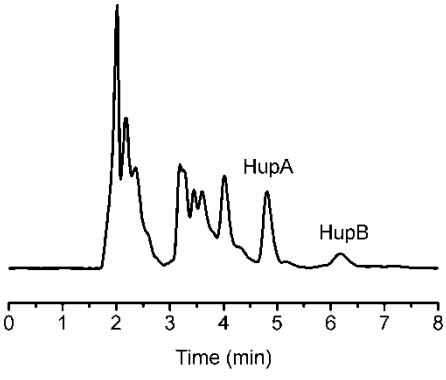


***Figure 2.*** HPLC analysis of the herb extract form *H. serrata*. Chromatographic conditions: column, Inertsil Ph-3 (250 mm × 4.6 mm ID, 5 μm); mobile phase, methanol-water (90:10, v/v); flow rate, 1.0 mL/min; temperature, 25°C; detection UV wavelength, 311 nm.

## Comparison of the Initial Separations Between XAD-4 LPLC and LLE

In the solvent extract of *H. serrata*, the low purity and concentration of HupA and HupB, as well as concomitant extraction of a large amount of non-polar components and polar impurities, however, are disadvantageous for subsequent chromatographic separation of HupA and HupB. In order to obtain high-purity HupA and HupB with additional chromatography methods, either LLE or adsorption separation might be useful to initially clean up the very crude feed in natural product isolation.

Figure 3 shows the profile of XAD-4 chromatography to capture HupA and HupB from the solvent extract of *H. serrata*. The fraction corresponding to both HupA and HupB was analyzed by HPLC and is shown in Figure 4A. Figure 4B presents HPLC analysis of the crude extracts obtained from LLE. The quantitative comparison of the results of these two methods is shown in Table 2. After the step of XAD-4 LPLC, the purities of HupA and HupB were about twice over those of LLE, respectively, and the recoveries were also much higher.

The result of LLE was not satisfactory. Its reason was assumed as follows: firstly, emulsification that often occurred during phase separation of LLE impaired the recovery of the products; secondly, in our study, multiple steps of liquid–liquid partition were adopted in order to get as much product as possible, but the purity certainly decreased at the same time. Finally, LLE was less favorable for the solvent extraction system where concentrations of



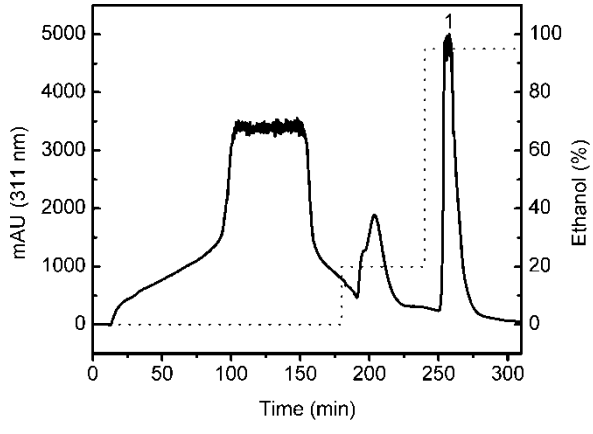


***Figure 3.*** The profile of adsorption separation of HupA and HupB from solvent extract by XAD-4 column LPLC. Chromatographic conditions: column size, 200 mm × 25 mm ID; sample loading, a 1.5 L of solvent extract (Sample B); flow rate, 10 mL/min; detection UV wavelength, 311 nm. Peak 1 represents both HupA and HupB.

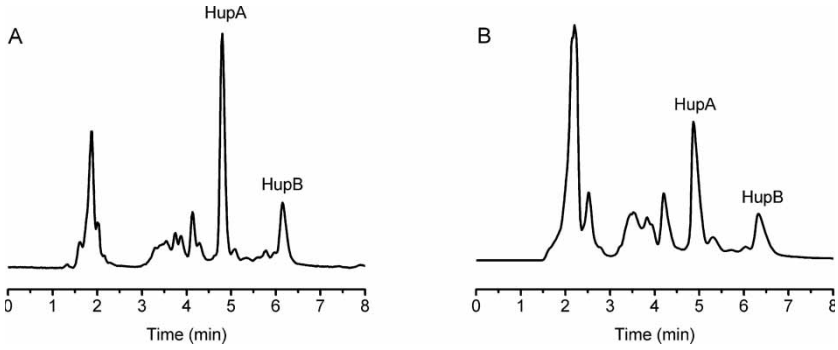


***Figure 4.*** HPLC analysis of crude extracts obtained from chromatographic fraction corresponding to peak 1 in Figure 3 (A) and LLE separation (B), respectively. Chromatographic conditions as in Figure 2.



desired products were very low. It is preferable to concentrate the solvent extract by evaporating before LLE, as described by Yu et al.;[8] however, this step may be both time- and energy-consuming. Moreover, the reproducibility of LLE was unsatisfactory, with the RSD of the recoveries and purities both over 15% for three batches.

Adsorption separation using XAD-4 was facile and efficient, with good batch-to-batch reproduciblility. The relatively large particle size of XAD-4 enabled it to operate under comparatively high flow-rate at low pressure, yet giving a high speed of separation. XAD-4 LPLC took about 6 h to complete a single run; however, the repeated LLE requires much more

***Table 2.*** Quantitative comparison of the results of XAD-4 chromatography and LLE separation of HupA and HupB from 1.5 L of the solvent extract

| | XAD-4 chromatography[a] | | LLE[b] | |
|---|---|---|---|---|
| | HupA | HupB | HupA | HupB |
| Amount (mg) | 18.7 | 8.5 | 14.4 | 6.3 |
| Purity (%) | 2.52 | 1.15 | 1.21 | 0.53 |
| Recovery (%) | 93.5 | 94.4 | 72.1 | 69.8 |
| Purification factor[c] | 14.0 | 14.4 | 6.7 | 6.6 |
| RSD of purity (%) | 3.82 | 4.15 | 15.1 | 15.6 |
| RSD of recovery (%) | 2.32 | 1.58 | 17.7 | 16.3 |
| Total amount of the dried crude extract (mg) | 742 | | 1190 | |

[a]Averaging the results of seven batches.
[b]Averaging the results of three batches.
[c]Purification factor = (Purity in the crude extract/Purity in the solvent extract).

time, depending on the speed of phase separation and the extent of emulsification. Additionally, 1.5 L of the solvent extract could easily be concentrated to about 200 mL in a single run without phase change. As a result, XAD-4 column LPLC was chosen for initial separation of HupA and HupB from the solvent extract of *H. serrata*.

## Comparison of the Final Separation Between the $C_{18}$ and PST Column LPLC

In the final purification, we compared the separation results of the commercial $C_{18}$ packing and the recently developed PST packing under their individual optimal operating conditions. The particle sizes of both media are larger than those of the HPLC media, but much smaller than those of traditional macroporous polymeric adsorbents. Therefore, it is expected to achieve a higher resolution of HupA and HupB on these two media than on XAD-4 under the low operating pressure.

Figure 5 is the separation profile of the $C_{18}$ column (A) and the PST column (B). The method using the PST column demonstrates a much higher resolution and better peak shapes of HupA and HupB. Table 3 is the quantitative comparison of the results of the two LPLC separations. Due to the serious peak tailing and the overlap of the peaks corresponding to HupA and HupB, the recoveries and purities for both HupA and HupB were low on the $C_{18}$ column. However, HupA and HupB were well resolved on the PST column. Using this effective LPLC method, HupA and HupB can be remarkably purified from 2.52% to 91.6%, and 1.15% to 92.3% with recoveries of 92.5% and 94.8%, respectively. Figure 6 shows the HPLC analysis of the fractions corresponding to HupA and HupB of the PST column LPLC, respectively.

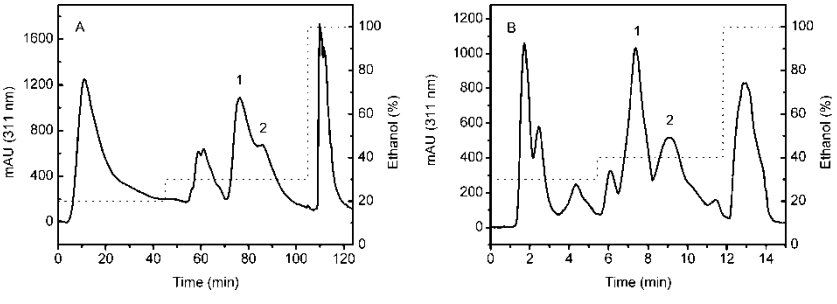


***Figure 5.*** The profile of chromatographic purification of HupA and HupB from crude extract by C18 column (A) and PST column (B). Chromatographic conditions: (A) column size, 100 mm × 16 mm ID; sample loading, 0.5 g of crude extract; flow rate, 2 mL/min; detection UV wavelength, 311 nm; (B) The flow rate was 12 mL/min, other conditions as in (A). Peak 1 and peak 2 represent HupA and HupB, respectively.

***Table 3.*** Quantitative comparison of the results of the C18 and PST column LPLC separation of HupA and HupB from the crude extract

| | C18[a] | | PST[b] | |
|---|---|---|---|---|
| | HupA | HupB | HupA | HupB |
| Purity (%) | 87.9 | 50.4 | 91.6 | 92.3 |
| Recovery (%) | 70.3 | 87.5 | 92.5 | 94.8 |
| Purification factor[c] | 27.9 | 76.1 | 36.7 | 82.4 |
| RSD of purity (%) | 9.77 | 8.52 | 1.64 | 1.77 |
| RSD of recovery (%) | 8.95 | 6.73 | 1.72 | 1.44 |

[a]Averaging the results of three batches.
[b]Averaging the results of seven batches.
[c]Purification factor = (Purity in the fraction of final LPLC separation/Purity in the crude extract).



The higher selectivity of the PST column may be the result of the presence of $\pi$-$\pi$ interactions between the aromatic-rich surface of this polystyrene-divinylbenzene resin and the pyridone ring of HupA and HupB; this specific interaction was mostly found in the separation of aromatic compounds on polystyrene resins.[19,20] On the $C_{18}$ packing, this interaction was absent, thereby reducing its selectivity. Moreover, the irreversible adsorption of the product and the impurities may occur on the $C_{18}$ medium surface, leading to low recovery of the products.

The regeneration of PST column was facile and rapid, and the batch-to-batch reproducibility was satisfactory, as shown in Table 3. Resulting from the irreversible adsorption, the separation on the $C_{18}$ column was from bad to worse after three batches of separation. Therefore, an extensive cleanup procedure should be required to restore its quasi-original state.

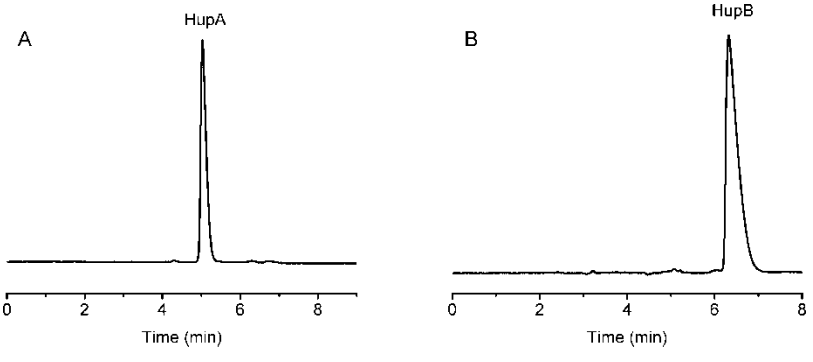


***Figure 6.*** HPLC analysis of the PST column chromatographic fractions corresponding to peak 1 (A) and peak 2 (B) in Figure 5B. Chromatographic conditions as in Figure 2.

The high viscosity generally hindered the use of ethanol in reversed-phase chromatography. The pressure drop of the $C_{18}$ column was over 0.5 MPa at a flow rate of 2 mL/min with ethanol-water as mobile phase, which made it difficult to improve the productivity by increasing the flow rate. However, the superior pressure-flow characteristics which resulted from the narrow size distribution enabled PST operated at a higher flow rate with significantly lower pressure. The pressure drop was still less than 0.2 MPa, even when the flow rate reached 12 mL/min. Therefore, the PST column undoubtedly has the advantages of rapid separation over the traditional $C_{18}$ column at LPLC mode, and in this case, the residence time on the PST column was less than 15 min, whereas it was more than 120 min on the $C_{18}$ column.

## Total Result of the Purification of HupA and HupB



There were minor impurities left in the purified fraction of HupA and HupB after the successive treatment with the XAD-4 and PST column LPLC operation. Therefore, the fractions of seven batches of the PST chromatography, corresponding to HupA and HupB, were respectively combined, and then directly crystallized and dried. The purities of both HupA and HupB can reach more than 98%, with the recoveries over 94% after a single step of crystallization. The structure of the obtained HupA and HupB was confirmed by IR, MS, and NMR spectroscopy. These data were identical to those reported in the literature.[1,7]

Table 4 shows the total results of HupA and HupB purification on the basis of seven batches of the PST chromatography. As demonstrated in Table 4, 78.1 mg of HupA and 35.9 mg of HupB were, respectively, obtained from 52.4 g of the solvent extracts (i.e., about 470 g of the dry herb powder), with the total recoveries of 82.8% and 84.3%. The yields on the basis of the

***Table 4.*** The total result of HupA and HupB purification.

| | Steps | | | |
|---|---|---|---|---|
| | Solvent extract | XAD-4 LPLC | PST LPLC[a] | Crystallization |
| Total (g) | 52.4 | 3.5 | – | – |
| HupA (mg) | 94.3 | 88.2 | 81.6 | 78.1 |
| Purity of HupA (%) | 0.18 | 2.52 | 91.6 | 98.2 |
| Recovery of HupA (%) | – | 93.5 | 86.5 | 82.8 |
| HupB (mg) | 42.6 | 40.3 | 38.2 | 35.9 |
| Purity of HupB (%) | 0.08 | 1.15 | 92.3 | 98.8 |
| Recovery of HupB (%) | – | 94.4 | 89.5 | 84.3 |

[a]HupA and HupB were separated after this step.

weight of the dry herb powder, about 0.017% and 0.0076% for HupA and HupB, respectively, are considerably higher than those reported in the literature, which were 0.01% for HupA and 0.0008% for HupB.[7,8]

This integrated process achieved rapid separation of HupA and HupB without using any time-consuming methods such as repeated LLE and re-crystallization, which were used in traditional process.[7–9] Compared with about 1 month as reported,[9] a single run here was greatly reduced, to 3–4 days (involving 2 days of solvent extraction). Both the XAD-4 and PST chromatography were easily performed using ethanol-water as mobile phase in step gradient elution mode under low pressure. The use of highly toxic solvents was avoided; therefore, the process can be considered as environmentally friendly.

## CONCLUSION

An integrated chromatographic process was set up for purification of HupA and HupB from *Huperzia serrata* using two polystyrene based resins, Amberlite XAD-4 and novel PST porous microsphere, as packing material, and ethanol-water as mobile phase. The initial separation with XAD-4 chromatography was able to remove a large amount of impurities and capture HupA and HupB from the solvent extract, and was much more efficient than multiple-step LLE. Compared with conventional $C_{18}$ chromatography, the subsequent PST chromatographic separation provides significantly better selectivity, lower operating pressure, and higher productivity in the final purification of HupA and HupB. After crystallization, highly purified HupA and HupB were obtained with the total recoveries of 82.8% and 84.3%, and the yields about 0.017% and 0.0076% on the basis of the weight of the dry herb powder.



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