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Simultaneously preparative purification of Huperzine A and Huperzine B from *Huperzia serrata* by macroporous resin and preparative high performance liquid chromatography

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ABSTRACT

Huperzine A (HupA) and Huperzine B (HupB) are natural alkaloids existed in *Lycopodium* plants. They both have potential clinical application for treating Alzheimer's Disease (AD). For the purpose of better utilizing the limited plant resources, a quick and low cost method to separate and purify HupA and HupB from *Huperzia serrata* (Thunb. ex Murray) was established in this paper. Low polarity macroporous resin SP850 was selected from eight kinds of resins during initial purification. Trifluoroacetic acid (TFA) was proved to be the best acid modifier reagent among all acids used in our experiment for improving separation. HupA and HupB were baseline separated on a C18 column by preparative high performance liquid chromatography (Preparative HPLC), the optimal gradient mobile phase system contained methanol increasing from 15% (v/v) to 35% (v/v) and 0.1% (v/v) TFA within the water. The purity of HupA and HupB obtained was 99.1% and 98.6%, respectively, and the total recovery for them was 83.0% and 81.8%, respectively.

1. Introduction

Huperzia serrata, one of the most common *Lycopodium* plants, has been used as a traditional medicine called *Qian CengTa* in China since the ancient time. Its original pharmacological action is to fix symptoms like contusions, strains, swelling, and schizophrenia [1]. After Huperzine A (HupA) and Huperzine B (HupB) (Fig. 1) were discovered in the *H. serrata* [2], attention was drew to the medical potential of *Lycopodium* alkaloids within the plants, especially their positive effects on learning and memory [3]. HupA has been proved to be a high selective and reversible acetylcholinesterase inhibitor (AChEI), and it has already been applied as a new drug for the treatment of Alzheimer's disease (AD) [4]. Compared with current drugs admitted by FDA for the treatment of AD: tacrine, donepezil, rivastigmine, and galantamine, HupA and its semi-synthetic derivative ZT-1 both perform relatively higher AChEI activity and longer-lasting drug action time, besides, they can penetrate smoothly through the blood–brain barrier and has much less butyrylcholinesterase (BuChE) activity, hence barely have side effects for human body [5,6]. HupB has also been proved to be a strong AChEI and performs a higher therapeutic index and a neuroprotective effect by attenuating hydrogen peroxide-induced injury [7]. Furthermore, it has also found that HupA and HupB are also effective for other cholinesterase-activity-related diseases, such as myasthenia gravis and vascular dementia [2,5].

However, the average contents of HupA and HupB are quite low in *Lycopodium* plants, approximately 0.2% and 0.08% in *H. serrata*. Thus, it is very difficult to obtain high purity of HupA and HupB, and the limitation cannot catch up with the increasing demand of HupA and HupB for clinical and research usage [8]. The similar structures of HupA and HupB make it much more difficult to separate the two alkaloids simultaneously [9]. Traditional steps like liquid–liquid extraction, silica gel column chromatography, and crystallization methods were frequently used for separating HupA, yet the HupB was always discarded [2,10,11]. Moreover, all these traditional processes required large amount of organic solvents and multiple time-consuming processes, with poor yield and recovery of HupA and HupB. Other studies focused on replacing for the plants resources, such as the cultivation of the *Huperzia* plant tissue [12,13], chemical synthesis of HupA and its analogs [14–17], and endophytic fungus fermentation [18]. All of these methods are potential resources, yet they are not applicable to feasible large scale production temporarily. To solve these problems and meet current demand, we aim to invent a simple purification process by macroporous resin and preparative HPLC.

In previous research, reverse-phase C18 has been applied in the determination of HupA and HupB [19–22]. In order to ameliorate the resolution and reduce the peak trailing, modifier reagents have to be added. These additives mainly include buffer salts like ammonium acetate [21,22], and organic amines like triethanolamine [19]. More investigations show that HupA and HupB is hard to be

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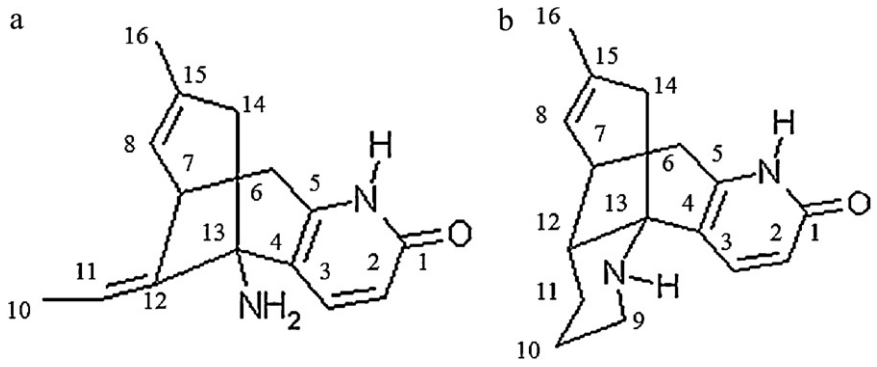

Fig. 1. The chemical structures of (a) Huperzine A and (b) Huperzine B.

separated simultaneously even triethylamine was added as modifier reagent, and the two alkaloid peaks cannot have baseline separation [23,24]. Based on the prior information, modifier reagents behave differently toward separation of certain compound. Consequently, choice of a suitable modifier reagent might also be a key factor for preparative HPLC separation. It has been reported that acids are also effective pH stabilizer, they can maintain the pH value of the solutions in proper range for the column [25,26]. Secondly, buffer salts have to be removed by other methods after the purified samples are collected, but most acids used can be removed easily, because they are volatile [27]. Till now, little study has adopted acids as the modifier reagents for separating HupA and HupB. In this paper, a well-improved method was successfully established to obtain high purity HupA and HupB. SP850 macroporous resin was initially selected for the pre-treatment, then trifluoroacetic acid (TFA), acetic acid, formic acid, and phosphoric acid were chosen to compare their influence on the separation of Hup A and Hup B. Through this method, highly pure HupA and HupB were obtained simultaneously, and the chemical structures of the purified compounds were verified by MS, ^{1}H NMR and ^{13}C NMR.

2. Experimental

2.1. Materials and reagents

Dry *H. serrata* herbs were purchased from AnGuo Chinese Medicine Market, Hebei. Huperzine A and Huperzine B standards were purchased from Tauto Biotechnology Corporation, Shanghai. Methanol and trifluoracetic acid (TFA) used for HPLC analysis and preparative liquid chromatography were of chromatography grade and purchased from Fisher Scientific Co. (Waltham, USA) and Merck Co. (Hohenbrunn, Germany) separately. Ultra-Pure water used for analytical and preparative HPLC was produced by Milipore Q System (Millipore, USA). All other chemicals and reagents were analytical grade and purchased from Beijing Chemical Factory (Beijing, China).

2.2. Preparation of the extract solution

Dry *H. serrata* herbs were homogenized in an analytical grinder. The smashed powder was added into hydrochloric acid solution (Solid/liquid = 1/10 (w/v), pH 2.0), the acid extract reaction was executed for 20 h by ultrasonic assistant method. The whole extract process was done at room temperature. After the first extraction, the filter residue was extracted for a second time in the same way, finally the acid filter solution was mixed together. The pH value of the obtained solution was adjusted to 9.0 by aqueous ammonia, preparing for submitting to the macroporous resin column chromatography and liquid–liquid extraction. The alkaloids content of the solution was determined by HPLC.

2.3. Selection of macroporous resins

For the prior treatment, the resin was soaked for 24 h with 100% ethanol, then washed with 4% HCl, 4% NaOH solution, and finally washed with deionized water. Macroporous resins HP20, ADS7, HPD100, SP850, HP2MG, HP2MGL, NKA, and HK801 were chosen for the static adsorption experiment. The preliminary selection of these resins was based on their adsorption and desorption capacities, as well as the ratio of desorption. 0.5 g of pretreated resin was put into three 250 mL air-tight Erlenmeyer flasks respectively. Then 100 mL of extract solution was added into each flask. The flasks were then shaken at 110 rpm for 24 h, at constant temperature of 298 K. After adsorption equilibrium was reached, the resin was desorbed with 100 mL of 90% (v/v) ethanol. Also, the flasks were shaken at 110 rpm for 24 h, at constant temperature of 298 K. The solutions after adsorption and desorption were analyzed by HPLC.

2.4. Purification by SP850 macroporous resin column chromatography

The macroporous resin column chromatography was carried out on a low-pressure glass chromatographic column (35 mm × 500 mm) filled with pretreated SP850 macroporous resin (Diaion High Porous Polymer SP-series, 0.315–1.25 mm particle size, Mitsubishi Chemical Industries Limited, Tokyo, Japan).

The separation by resin-based column was executed as following: first, extract solution (10 L, pH 9.0) was loaded onto the column and adsorbed at a flow rate of 5 BV/h. After adsorption, the column was first washed with 6 BV deionized water, and then eluted with 3 BV of 10% (v/v) ethanol solution to remove the polar impurities, the crude extract was eluted with 6 BV of 70% (v/v) ethanol solution at a flow rate of 5 BV/h. At last, the non-polar contaminants were eluted with 3 BV of 100% ethanol. All the fractions were collected precisely and quantitatively analyzed by HPLC. The crude extract was dried by rotator evaporator at 40 °C.

2.5. Selection of acid modifier reagents for separation of HupA and HupB

The mobile solvent system consisting of pure methanol (mobile phase A) and pure water (mobile phase B) was initially tested. The optimized ratio was set as methanol:water = 18:82 (v/v) in the following experiment. Four kinds of acids: 0.5 mL TFA, 0.5 mL acetic acid, 0.5 mL formic acid, and 0.5 mL phosphoric acid, were added into 499.5 mL pure water, each was used as improved mobile phase B for the analysis. The pH values of improved mobile phase B were measured before use. Sample injected is standard solution

consisting of 60 mg/L HupA and 30 mg/L HupB. For each different acid solvent system, the sample was injected for three times. The retention time and the separation resolution were recorded and compared.

2.6. Analytical HPLC

The analytical HPLC equipment used was a Shimadzu LC-20AT system with two LC-20AT solvent delivery units, a SPD-M20A DAD detector, a SIL-20A auto sampler, a CTO-10ASVP column oven, a LC solution workstation (Shimadzu, Kyoto, Japan) and an analytical reversed phase C18 column (4.6 mm × 250 mm, 5 μm, Diamodsil™). The solvents system included mobile phase A and B in the ratio of 18:82 (v/v). Mobile phase A was pure methanol. In the acids selection experiment, each improved mobile phase B was constituted of 0.1% (v/v) TFA, 0.1% (v/v) acetic acid, 0.1% (v/v) formic acid, and 0.1% (v/v) phosphoric acid separately as modifier reagents. Mobile phase B with 0.1% (v/v) TFA was used for the analysis in all other experiments. The column oven temperature was set at 30 °C. The flow rate was 1 mL/min, and 10 μL portions were injected into the column. Both HupA and HupB were detected by UV 308 nm.

2.7. Preparative HPLC

A glass chromatographic column (30 mm × 800 mm, H&E Co., Ltd. Beijing) filled with YMC C18 packing (50 μm particle size, Greenherbs Science & Technology Department Corporation, Ltd.) was used for the low pressure chromatographic separation before preparative HPLC. The crude extract was dissolved in 20% ethanol as the sample solution. This separation process was executed according to Li [24] as following: 20% (v/v) ethanol was used to elute for the first 60 min, then 30% (v/v) ethanol was used to elute the products for the next 60 min, at last, 100% (v/v) ethanol was used to wash the impurity left on the column for 60 min. All fractions are collected and analyzed by HPLC, the concentrated extract was dried as method above, then submit to preparative HPLC.

The preparative high performance liquid chromatography equipment used was a Waters Prep 4000 liquid chromatography system equipped with a fluid handling unit (pump heads), controller (for solvent gradient, flow rate, external events, and sparging process), a 2487 dual-wavelength absorbance detector with a preparative cell (Waters, Milford, MA), an Empower workstation (Waters, USA) and a reversed phase C18 column (19 mm × 300 mm, 7 μm, Symmetry Prep™). The preparative HPLC separation was performed as follows: mobile solvent system was consisted of 0.1% (v/v) TFA in water and 15% (v/v) methanol in the first 20 min, it then changed linearly over 30 min to 35% methanol, and remained unchanged in the last 10 min. The injection volume was 10 mL. The flow rate was 8 mL/min, and the detection wavelength was 308 nm for monitoring HupA and HupB. Peak fractions of HupA and HupB were collected manually according to the preparative HPLC chromatogram. The fractions were concentrated by the rotary evaporator, and processed using a vacuum freeze-drying machine.

2.8. MS and NMR

Electrospray ionization mass spectra (ESI/MS) and nuclear magnetic resonance (NMR) spectra were obtained by analysts at the Center of Analysis, Beijing University of Chemical Technology. A Micromass Quattro-Primier mass spectrometry was used with an ion source temperature of 100 °C. The spectrum was scanned in the range of *m/e* 50–800 in positive ion mode, and the sample was dissolved in methanol. The ^{1}H and ^{13}C NMR spectra were obtained by a Bruker high-resolution AV600 NMR spectrometer at 600 MHz (Bruker Biospin Corporation, USA). The samples were dissolved in $CDCl_3$, and the chemical shifts were reported in relation to the resonance peak of TMS chemical shift at $\delta = 0$ ppm as reference.

3. Results and discussion

3.1. HPLC analysis

A schematic of the purification process is depicted in Fig. 2. The chromatograms of standard HupA, HupB and each step fraction during the workflow are shown in Fig. 3. The standard samples injected concentration are 20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L, 100 mg/L for HupA, and 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L, 50 mg/L for HupB. Based on the linear regression analysis of the peak area responses

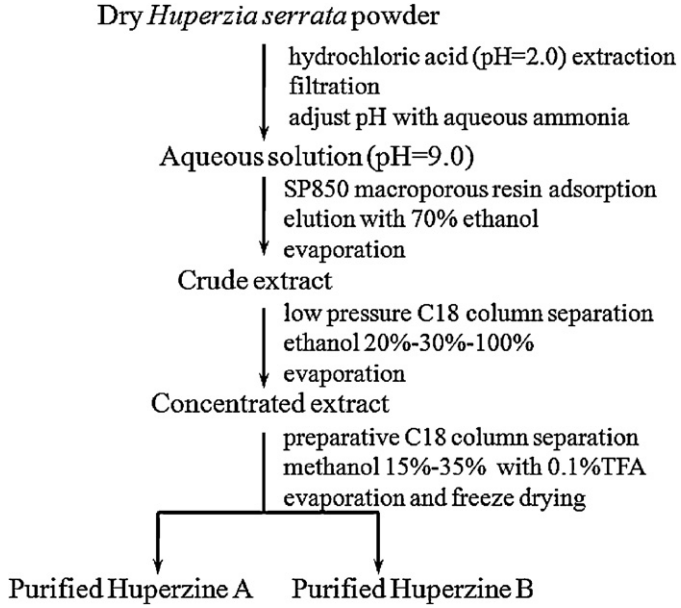

Fig. 2. The schematic of the purification workflow for Huperzine A and Huperzine B from *H. serrata* is shown. The fractions of each step: aqueous solution, crude extract, concentrated extract, and the purified products are all detected by analytical HPLC.

Table 1
Comparison of purification efficiency capacities of HupA and HupB among different purification methods.

Purification methods	Purity (%)		Recovery (%)		Total recovery (%)
	HupA	HupB	HupA	HupB	
Macroporous resin SP850	2.03	0.91	90.1	93.2	91.3
Liquid–liquid extraction[a]	1.86	0.82	70.1	72.0	71.0
Ion resin D72[b]	1.66	0.80	27.6	25.3	26.4

[a] Dates for LLE are carried out by extracting the base solution with chloroform for two times, the extracts are then dried and determined by HPLC.
[b] Dates for D72 resin based chromatography are carried out in the same condition as SP850 which is described in Section 2.4. All the purification experiments were at least repeated for five times.

(y) versus the theoretical concentration (x), the standard equation is: $y = 22{,}646x - 12{,}2153$, $r^2 = 0.9998$ (HupA); $y = 20{,}157x + 3591.2$, $r^2 = 0.9998$ (HupB). The correlation coefficient demonstrated linearity of the method over the concentration range analyzed.

3.2. Selection and separation by macroporous resin chromatography

Macroporous resins, ion exchange resins and traditional extraction with chloroform were tested and compared in the preliminary experiment. As a result, macroporous resin was proved to be superior than the other two approaches, it has better recovery and purification rate (Table 1). For further study, eight macroporous resins are chosen to test their adsorption and desorption capacity. The results are shown in Table 2. The adsorption capacity of HP20, HPD100, and SP850 for both HupA and HupB are higher than other resins. While the desorption capacity of HPD100 is lower than HP20 and SP850, especially for HupB. Considering both adsorption and desorption properties, the SP850 resin is finally selected for further investigations. SP850 exhibits the best adsorption and desorption capabilities because it has similar polarity with the low polarity HupA and HupB, also it has larger surface area and smaller pore size [28], thus make it suitable for purifying small low polarity alkaloid molecular. After concentration by SP850 resin, the purity of HupA and HupB in the crude extract increased from 0.02% and 0.0085% to 2.03% and 0.91%, the extract was enriched for more than 100 times compared with the original extract. The HPLC chromatograms before and after purified by SP850 resin are shown in Fig. 3b and c.

3.3. Selection of the acid modifier reagent

Methanol and acetonitrile are most frequently used organic reagent; it is reported that methanol give significantly better peak shape than acetonitrile on reversed-phase column [29], it is also relatively cheaper, hence we choose methanol as organic solvent in the mobile systems. Before acid modifier reagents were added, it is confirmed that HupA and HupB were eluted in poor retention and peak shape, and changing the ratio of methanol and water have little amelioration. After the acids were included in the water phase, HupA and HupB can be separated, see the chromatograms depicted in Fig. 4a–d. However, the same concentration of 0.1% acetic acid, formic acid, phosphoric acid, and TFA behave differently in their abilities for improving the separation efficiency. Compared with other acids, TFA exhibited the best selection and resolution. Adding acetic acid, formic acid, and phosphoric acid, the trailing problems remain existed and the peak is not quite symmetrical, in addition, the time interval between two alkaloids is limited. Only by adding TFA, an ideal peak shape can be obtained, and the time interval between HupA and HupB is extended to a satisfactory degree, which makes it possible to amplify to the preparative scale. The retention time selectivity factor (α), the retention factor (k'), and the resolution factor (Rs) between HupA and HupB are calculated and compared in Table 3. The result demonstrated that TFA performs better than other acid modifiers, within the proper range of $\alpha > 1.05$, $1 < k' < 10$, $Rs > 2$. The different pH values of the acids (Table 3) might account for the result. With the increase of acidity, the retention time is deferred, and the time interval between HupA and HupB is extended. In lower pH value environment the basic solutes are fully protonated, the residue silanol groups on the solid phase are neutral, thus they might have less electrostatic interaction with each other [26,27]. Unlike other widely used acid modifiers, TFA can adjust the pH value of solutions to a lower pH with less amount. In addition, it can also function as ion-pair reagents to control the selectivity and resolution of small ionizable chemicals [26]. In conclusion, we choose TFA as the suitable modifier reagent for further preparative separation.

Table 2
The adsorption and desorption capacities, and desorption ratio of Huperzine A and Huperzine B on different kinds of macropourous resins at 298 K.

Adsorbent	Adsorption capacity (mg/L)		Desorption capacity (mg/L)		Desorption ratio (%)	
	HupA	HupB	HupA	HupB	HupA	HupB
HP20	9.34 ± 0.12	3.82 ± 0.02	8.54 ± 0.11	3.42 ± 0.08	91.37 ± 2.22	89.74 ± 2.12
ADS7	4.60 ± 0.07	2.53 ± 0.12	3.23 ± 0.09	0.96 ± 0.11	70.16 ± 2.92	37.98 ± 4.12
HPD100	7.85 ± 0.05	3.53 ± 0.12	6.67 ± 0.11	0.96 ± 0.02	84.87 ± 2.01	27.27 ± 1.52
SP850	10.3 ± 0.03	4.00 ± 0.05	9.65 ± 0.08	3.73 ± 0.03	93.60 ± 1.63	93.30 ± 1.88
HP2MG	4.65 ± 0.03	2.60 ± 0.02	3.69 ± 0.12	0.96 ± 0.02	79.31 ± 1.89	37.04 ± 0.94
HP2MGL	4.89 ± 0.15	2.66 ± 0.22	3.78 ± 0.07	0.96 ± 0.04	77.17 ± 1.98	36.21 ± 1.52
NKA	4.97 ± 0.11	2.75 ± 0.10	4.33 ± 0.14	0.96 ± 0.07	87.01 ± 2.12	34.99 ± 3.82
H2K801	5.62 ± 0.14	3.06 ± 0.09	4.11 ± 0.09	0.96 ± 0.02	72.00 ± 1.33	31.43 ± 1.90

Table 3
Comparison of different acid modifier reagents for the retention and resolution of HupA and HupB.

Acid type	pH value	Retention time (min)		k'		$\alpha_{A/B}$	Rs
		HupA	HupB	HupA	HupB		
0.1% acetic acid	3.61	3.728	5.548	0.798	0.209	3.835	0.795
0.1% formic acid	2.67	6.761	10.182	2.126	1.076	1.976	3.252
0.1% phosphoric acid	2.05	7.978	12.204	2.805	1.488	1.886	4.205
0.1% trifluoracetic acid	1.87	13.209	21.865	5.795	3.137	1.862	8.277

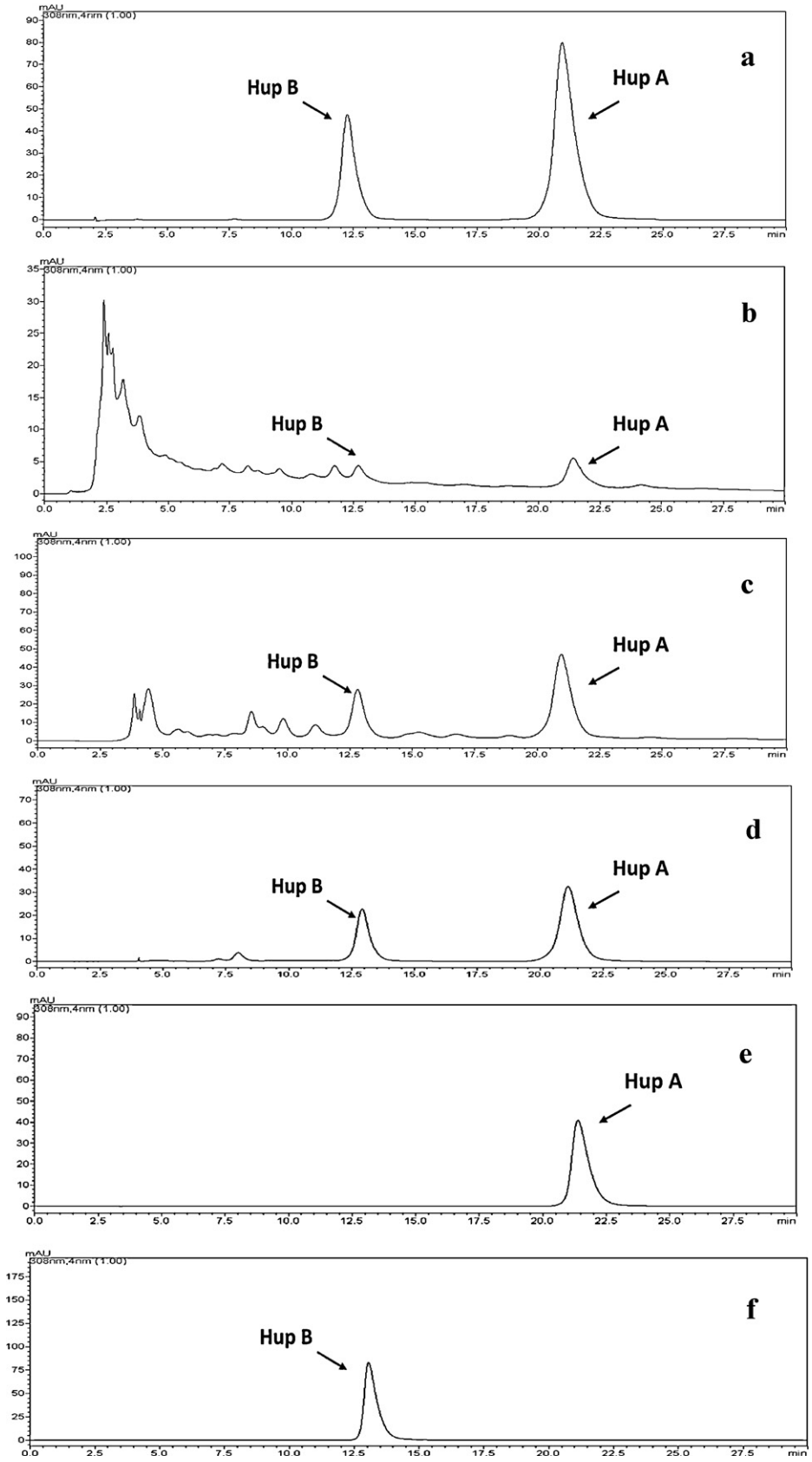

Fig. 3. The analytical HPLC chromatograms are illustrated in the sequence of (a) HupA 100 mg/L and HupB 50 mg/L mixed standard; (b) the original extract solution; (c) the crude extract purified by SP850 resin; (d) the concentrated extract before submitted to preparative HPLC and (e) (f) the purified HupA, HupB collected after preparative HPLC. Analytical HPLC conditions are shown in Section 2.6.

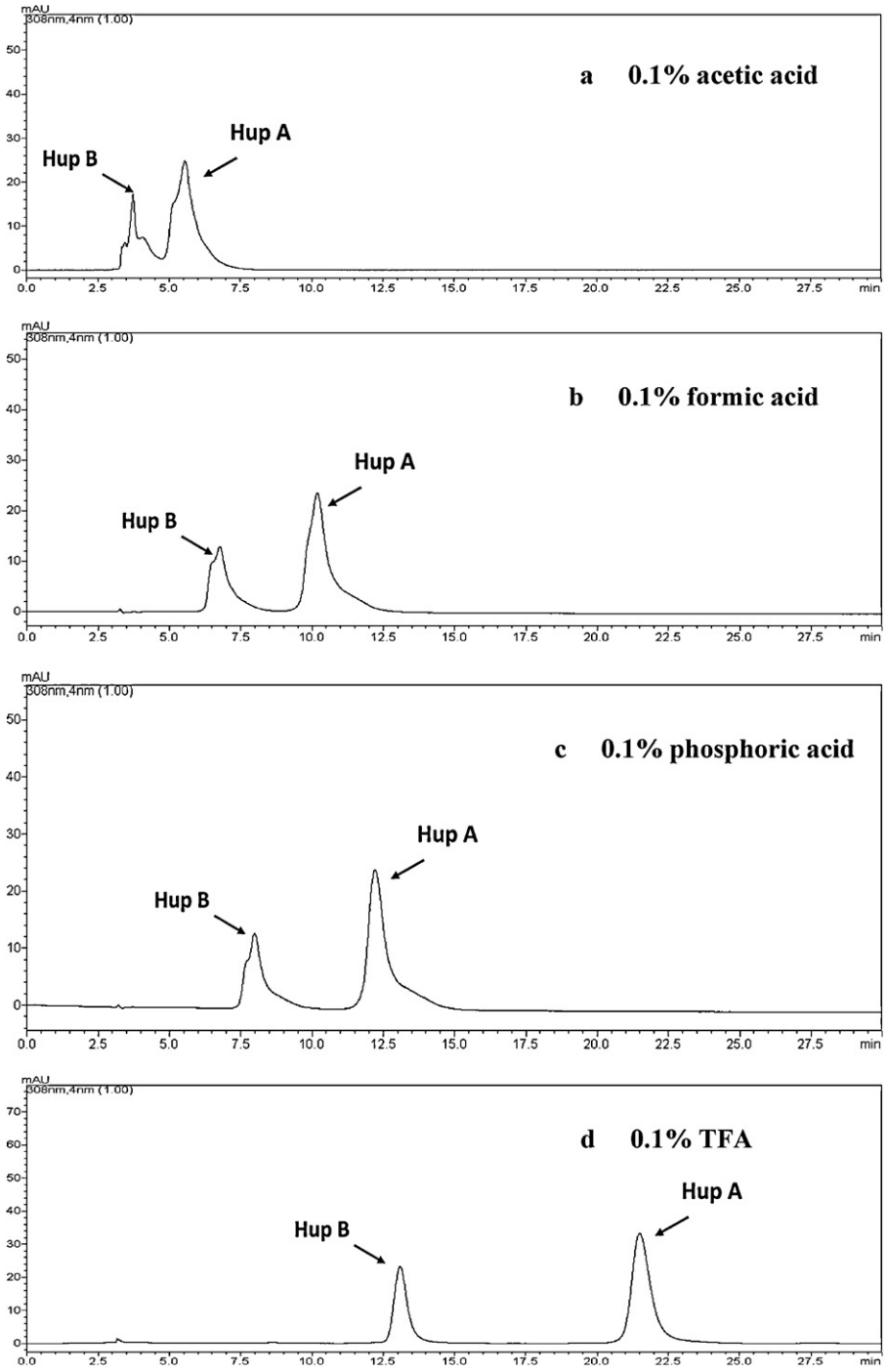

Fig. 4. The analytical HPLC chromatograms of different kinds of modifier solvent system are shown in the sequence of (a) 0.1% acetic acid, (b) 0.1% formic acid, (c) 0.1% phosphoric acid and (d) 0.1% TFA; samples injected are mixed standard with HupA 60 mg/mL and HupB 30 mg/mL. Analytical HPLC conditions are shown in Section 2.6.

3.4. Preparation of high purity HupA and HupB through preparative HPLC

In order to increase the loading amount, a purification step by low pressure C18 column was executed. In this step, most non-polarity and strong polarity impurities are removed by water and pure ethanol separately, HupA and HupB are co-eluted by the low content ethanol. The extract was enriched again for about ten times, with the purity of 54.6% and 22.5% separately for HupA and HupB, with an ideal recovery of 93.5% and 92.2%.

To further optimize the mobile system, TFA concentrations ranging from 0.01% (v/v) to 0.2% (v/v) were tested during preparative separation. The pH value of 0.01% trifluoracetic acid was 2.85, under which condition both HupA and HupB peaks were unsatisfactory, When TFA concentration grew beyond 0.1%, the selectivity and resolution became much better. Although the larger loading amount extended the peak width, HupA and HupB are well baseline separated. The result fit with the prediction we made that lower pH enhance the separation resolution of alkaloids. During preparative scale separation, adding more TFA decreased the pH value of the solvent system, the retention time is deferred. In lower pH condition, the protonation process of the ionizable alkaloids is also much faster. Besides, by shielding the polarity groups on the stationary phase, TFA can greatly reduce the interaction between the

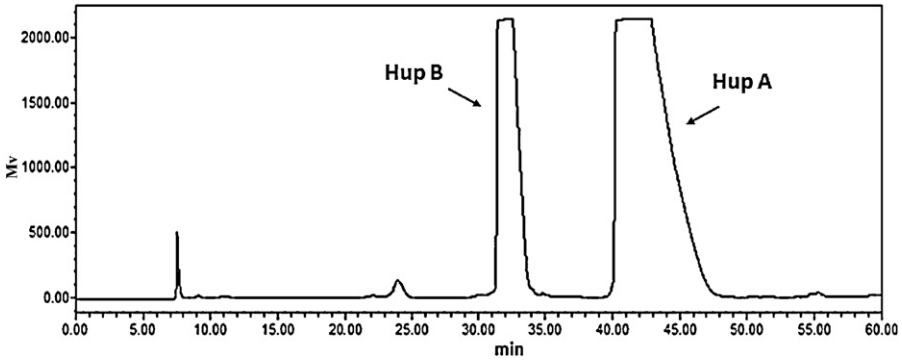

Fig. 5. The preparative HPLC chromatogram of concentrated *H. serrata* extract. Preparative HPLC column: a reversed phase C18 column (19 mm × 300 mm, 7 μm, Symmetry Prep™). Preparative HPLC conditions are shown in Section 2.7.

alkaloids and the dissociated silanol groups on solid phase. However, extremely low pH will be harmful to the solid medium. Consequently, 0.1% (v/v) TFA concentration is appropriate, for the actual pH of the mobile solvent system is about 2, and the separation time is relatively short.

After the gradient optimization, HupA and HupB are baseline separated with satisfactory resolution as depicted in Fig. 5, the gradient condition was described in Section 2.7. All the fractions of the peaks in the chromatogram are collected and analyzed initially, through comparation with the standard HPLC (see Fig. 3a and b). The main two peaks are initially verified as HupB and HupA chronologically. The collected purified products are dried by the rotator evaporator at 40 °C. Through the whole chromatography procedure, from 700 g herb powders, we finally obtain 105 mg HupA and 49 mg HupB with the purity of 99.1% and 98.6% separately. The recovery of HupA and HupB in preparative HPLC step is 94.3% and 95.8% separately, and the total recovery of the process is 83.0% and 81.8% for HupA and HupB, respectively. The HPLC chromatograms of concentrated extract before preparative HPLC, purified HupA and HupB after the separation are shown in Fig. 3d–f, respectively.

3.5. Confirmation of purified HupA and HupB

In order to make a further chemical structure identification of HupA and HupB, the collected products are submitted to the MS and NMR analysis. The mass spectrums for HupA and HupB, see Fig. 6a and b, were obtained and showed clear quality.

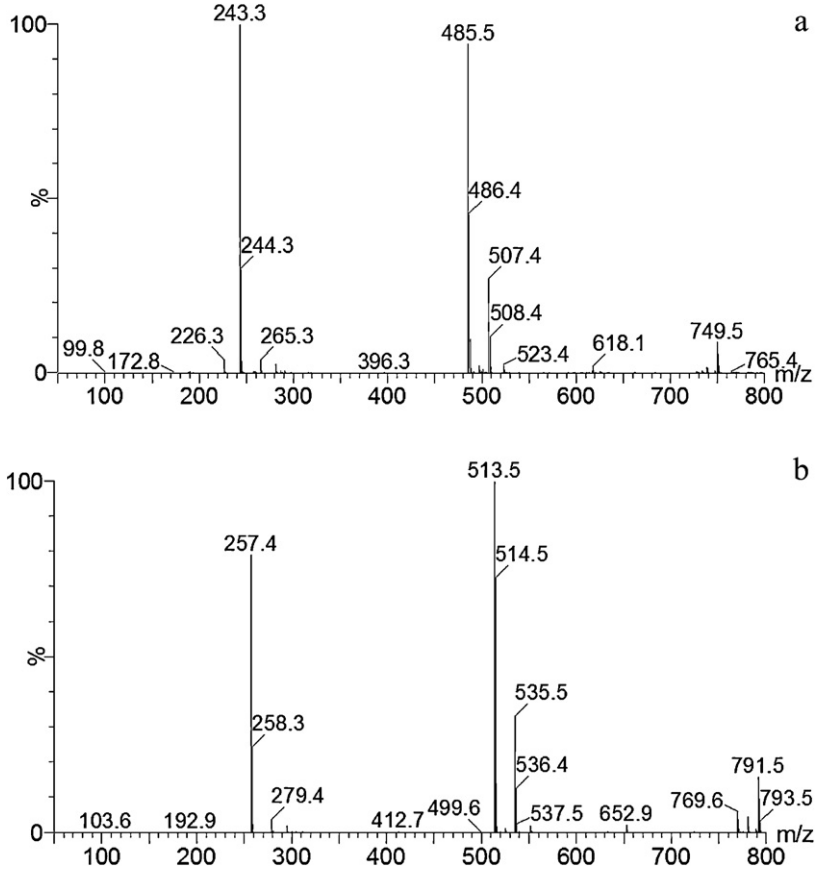

Fig. 6. Mass spectrograms of (a) HupA and (b) HupB purified by preparative HPLC.

Huperzine A: The peaks with *m*/*z* 243.3 and *m*/*z* 265.3 in Fig. 6a are confirmed to be $[M+H]^+$ and $[M+Na]^+$. Huperzine B: The peaks with *m*/*z* 257.4 and *m*/*z* 279.3 in Fig. 6b are confirmed to be $[M+H]^+$ and $[M+Na]^+$. The peaks *m*/*z* 485.5, *m*/*z* 507.4 in Fig. 6a, and *m*/*z* 513.5, *m*/*z* 535.5 in Fig. 6b are confirmed to be $[2M+H]^+$ and $[2M+Na]^+$ for HupA and HupB, respectively. Compared with the previous publications [2,30] the MS spectral data is coincident, also the ^{1}H NMR and ^{13}C NMR spectral data (provided in the supplement materials Table S1.) are coincident, the purified compounds obtained by preparative HPLC were confirmed as Huperzine A and Huperzine B.

4. Conclusion

Separation and purification by macroporous resin combining with preparative HPLC is applicable to concentrate low content of HupA and HupB simultaneously from the natural plants. Low polarity resin SP850 has superior capacity to purify HupA and HupB in the initial step. TFA is confirmed as an important additive to maintain the low pH mobile system, thus a satisfactory separation resolution can be obtained in the preparative HPLC process. The instruments and reagents used in the process are regular, environment-friendly and feasible for amplifying to larger scale. HupA and HupB with the high purity of above 98% can be obtained simultaneously after preparative separation on a regular C18 column, the total recovery is up to 80%. In conclusion, this method is applicable to satisfy fast production of HupA and HupB, and possible for improvement of industrial utilization of the *Lycopodium* plants. Meanwhile, it provides idea and valuable practical experience to the purification of natural products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.07.019.

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