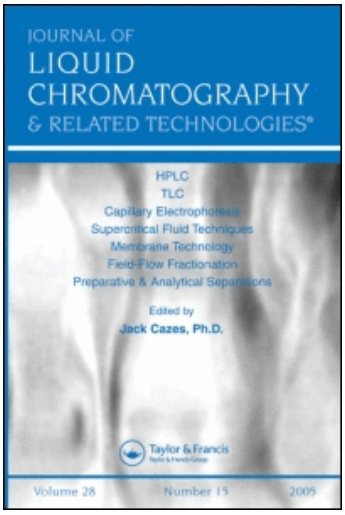


**Journal of Liquid Chromatography & Related Technologies**
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# Effect of Mobile Phase on Loading Mass for Preparative Separation of Sanguinarine and Chelerythrine on a Reverse Phase Column

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Online publication date: 05 November 2004

**To cite this Article** Zhang, Fei , Chen, Bo , Luo, Xu-Biao , Zhu, Xiao-Lan and Yao, Shou-Zhuo(2005) 'Effect of Mobile Phase on Loading Mass for Preparative Separation of Sanguinarine and Chelerythrine on a Reverse Phase Column', Journal of Liquid Chromatography & Related Technologies, 27: 10, 1491 — 1505
**To link to this Article: DOI:** 10.1081/JLC-120034087
**URL:** http://dx.doi.org/10.1081/JLC-120034087

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 10, pp. 1491–1505, 2004

# Effect of Mobile Phase on Loading Mass for Preparative Separation of Sanguinarine and Chelerythrine on a Reverse Phase Column

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## ABSTRACT

Optimization of the mobile phase focusing on loading mass for the separation of sanguinarine and chelerythrine by a preparative high performance liquid chromatograph (prep-HPLC) was investigated. The effects of the mobile phase with modifiers (ion pair, disodium hydrogen phosphate, and triethylamine) on loading mass were compared. Finally, disodium hydrogen phosphate buffer was selected to optimize. The optimum HPLC mobile phase was 0.05 mol/L disodium hydrogen phosphate buffer and acetonitrile (MeCN) (70/30, v/v) (pH = 3–3.5, adjusted by $H_3PO_4$). A larger loading mass and volume were obtained in the shorter time. Furthermore, it had been found that the general ion

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DOI: 10.1081/JLC-120034087 1082-6076 (Print); 1520-572X (Online)

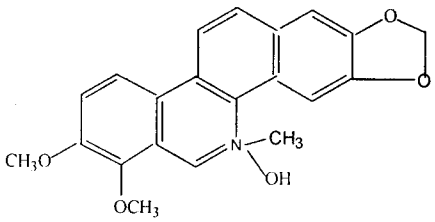
pair RP-HPLC for alkaloids analysis was not suitable for preparative separation for the components because of its low sample size.

*Key Words:* Sanguinarine; Chelerythrine; Prep-HPLC; Crude alkaloids; Loading mass.

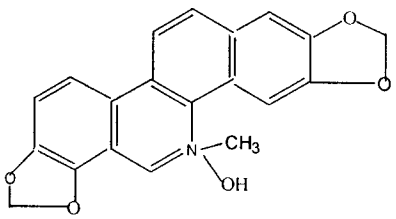
## INTRODUCTION

Sanguinarine and chelerythrine (Fig. 1) are benzophenanthridine alkaloids obtained from plants such as *Chelidonium majus* L.,[1] *Eomecon chionantha* Hance,[2] and *Macleaya cordata* (Willd) R. Br.[3] Recently, it has been found that they have antiseptic, and antitumour activities.[4,5] The isolation and preparation of the high purity components have an important significance.[6]

For the separation of sanguinarine and chelerythrine, a number of chromatographic methods such as reverse-phase HPLC,[7–9] normal-phase HPLC,[10] thin-layer chromatography (TLC),[2,10] and capillary electrophoresis (CE)[11] have been developed. However, the methods were used for analysis. The preparative separation with HPLC for the components has seldom been reported.




sanguinarine




chelerythrine

***Figure 1.*** Molecular structures of sanguinarine and chelerythrine.





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For preparative isolation of components with HPLC, some parameters must be investigated and optimized. For example, the throughput, sample size and loading factor, production rate, and so on, are the key factors of preparative high performance liquid chromatograph (prep-HPLC).[12–18] The mobile phase can affect the factors significantly. In general, analytical HPLC is the precursor of prep-HPLC.[12,17] However, the optimization of the experimental parameters in prep-HPLC cannot be completed simply based on the analytical HPLC. A number of analytical conditions are not suitable to scale up to prep-HPLC. For alkaloid analysis, the most universal conditions are that ion pair reagents are used as mobile modifiers of analytical HPLC.[19,20] The alkaloids can be separated on low sampling mass.

In this paper, the optimum mobile phase for preparative separation on reverse phase columns for sanguarine and chelerythrine is proposed. The effects of the mobile phase with modifiers (ion pair, disodium hydrogen phosphate, and triethylamine) on loading mass were compared. Finally, the buffer was selected. This method can be used as a guide to scale up the industrially preparative separation of sanguinarine and chelerythrine.



## EXPERIMENTAL

### Materials and Chemicals

The fruits of *M. cordata* (Willd) R. Br. were purchased from Changde, P.R. China. The macroporous resin HPD-100 was obtained from the Cangzhou Bon Chemical Co., Ltd. (Hebei, P.R. China) and packed in the glass column (inner diameter: 2.5 cm). The standards for sanguinarine and chelerythrine were purchased from Sigma Corporation (P.R. China) and dissolved with methanol.

Water, acetonitrile, and methanol (Shanghai, P.R. China) of HPLC grade were used for HPLC analysis and prep-HPLC. Formic acid, phosphoric acid, acetic acid, disodium hydrogen phosphate, triethylamine of analytical grade were used as mobile phase modifiers. Reagent-grade sulfuric acid was employed for crystallization. Ion pair reagent (B-7) was obtained from Waters, USA. All solvents used for HPLC were filtered (0.45 μm) and ultrasonically degassed before use.

### Instruments

Analyses of samples were performed on a Waters (Milford, MA) alliance 2695 liquid chromatographic system, interfaced to a 996 PAD detector



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equipped with a Johnson (Dalian, P.R. China) spherigel analytical column (4.6 × 250 mm), packed with 5 μm $C_{18}$ silica. The mobile phase consisted of 0.05 mol/L disodium hydrogen phosphate buffer and acetonitrile (65/35, v/v) (pH = 3–3.5, adjusted by $H_3PO_4$). A Spherisorb CN column (5 μm, 200 × 4.6 mm) was also used. The mobile phase consisted of acetonitrile and triethylamine aqueous solution (0.025 mol/L, pH = 3, adjusted by $H_3PO_4$) (28 : 72, v/v). In all analytical conditions, the flow rate was 1 mL/min, the column temperature was 30°C, and UV spectra was recorded at 270 nm.

The prep-HPLC consisted of a Waters prep-LC 4000 system, and a Waters 2487 Dual Absorbance Detector. The data obtained were recorded and treated with Millennium$^{32P}$ chromatographic software (Waters). The preparative experiments were performed in a 300 × 7.8 mm I.D. semipreparative column (Prep Nova-pak® HR $C_{18}$ Waters). The column temperature was at ambient temperature. The isocratic mobile phase, consisting of 0.05 mol/L disodium hydrogen phosphate buffer and acetonitrile (70/30, v/v, pH = 3–3.5), was pumped at 2.5 mL/min.

## Preparation of Crude Alkaloids

1000 g of fruits of *M. cordata* (Willd) R. Br. were extracted with 0.25% (v/v) sulfate acid aqueous solution for 1 hr at 90°C three times. After filtration, the filtrate was cooled till the temperature was 50°C. Then, sodium hydroxide was added until the pH values were about 12–13. The precipitates were produced. After filtration, the residue was dried in a vacuum drying oven. The dried residue (about 20 g) was reflux extracted for 1 hr with 80 mL of methanol (80 mL). The extract solution was then immediately filtered. The process was repeated three times. The filtrate was combined and vaporized till the volume was about 20 mL. After the solution cooled, 2 mL of sulfuric acid was added to the solution, the precipitates were produced. After cooling and filtration, the residue was dried and the crude alkaloids were obtained.

A 200 mL aliquot of the macroporous resin was packed in a glass column (length: 50.0 cm, inner diameter: 2.5 cm) and immersed with methanol for 2 hr, washed with 500 mL of de-ionized water. Five grams of crude alkaloids was ultrasonically treated with 150 mL of water for 15 min. After filtration, the precipitates were ultrasonicated with 50 mL of water for 10 min, and then filtrated. The solution was combined and passed through the column at a flow-rate of 2 mL/min. Then the column was desorbed sequentially with 1000 mL of 20%, 250 mL of 60% aqueous methanol solution, and 1000 mL of ethanol solution, and the desorption solution, which contained sanguinarine and chelerythrine, was concentrated with a rotary evaporate instrument and





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evaporated to dryness. The materials containing sanguinarine and chelerythrine for preparative chromatography were obtained.

## RESULTS AND DISCUSSION

### Refined Crude Alkaloids

In crude alkaloids of fruits of *M. cordata* (Willd) R. Br, there are also other alkaloids such as protopine and allocryptopine except sanguinarine and chelerythrine. In order to reduce the interference of other alkaloids to the separation of two alkaloids, a number of pre-purification methods such as strong acid cation-exchange resins and silica gel used as solid phase had been investigated. Due to strong polarity, sanguinarine and chelerythrine were seldom eluted with the strong acid cation-exchange resins in any case. With silica gel,[3] chloroform, methanol, and acetic acid (96 : 4 : 0.2 v/v/v) were used to elute these alkaloids. There was some improvement, but the yield was low and the elution of chloroform was more hazardous compared with the macroporous resins. Therefore, according to the literature,[21,22] a kind of macroporous resin was employed and the results were satisfactory.

The aqueous methanol and ethanol were used to wash the column to obtain the mixture of sanguinarine and chelerythrine. The desorption solution, which contained sanguinarine and chelerythrine, was concentrated with a rotary evaporate instrument. Through analyzing the mixtures obtained from different desorption solutions, it had been found that protopine and allocryptopine had been washed out from the column with low percentage aqueous methanol. Sanguinarine and chelerythrine had been eluted with ethanol. Although, the complete separation of sanguinarine and chelerythrine by this method was impossible, protopine and allocryptopine could be removed from crude alkaloids. Figure 2 shows the chromatograms of crude alkaloids before and after treatment by macroporous resin. This was very helpful for the purification of sanguinarine and chelerythrine using prep-RP-HPLC. After pre-purification, the total contents of sanguinarine and chelerythrine achieved 70%. This product was used as the raw material for the purification by prep-RP-HPLC.

### Choice of Preparative Separation Conditions

For the choice of the optimum preparative separation conditions, the experiments such as relationship between sample size with separation, etc. were completed on analytical columns.





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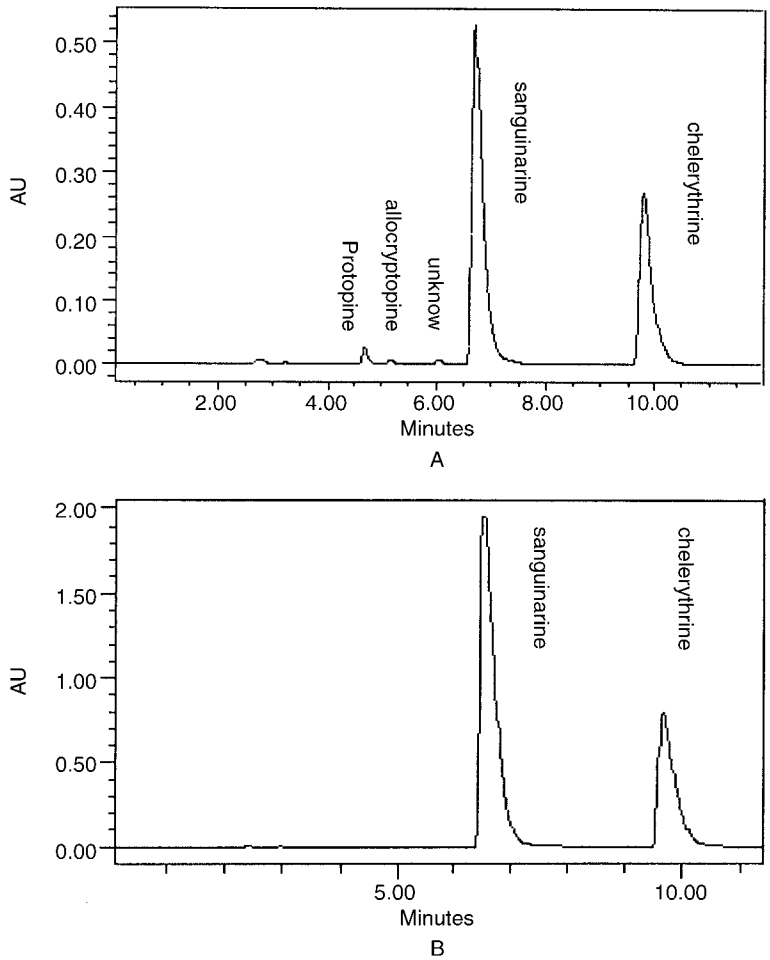


***Figure 2.*** Chromatograms of crude alkaloids before (A) and after (B) treatment by macroporous resin. Conditions: Johnson $CB_{18B}$ column, 4.6 × 250 mm, 5 μm; the mobile phase consisted of 0.05 mol/L disodium hydrogen phosphate buffer and acetonitrile (65/35, v/v) (pH = 3–3.5, adjusted by $H_3PO_4$); UV 270 nm; injection volume: 5 μL.

Sanguinarine and chelerythrine could be separated under different RP-HPLC conditions. The HPLC conditions for the separation of sanguinarine and chelerythrine are listed in Table 1. Figure 3 shows the chromatograms obtained from different chromatographic conditions. From the results, two alkaloids can be separated completely on an analytical level. However, some of conditions are not

***Table 1.*** Various HPLC conditions for two alkaloid separation.

| Condition number | Column | Mobile |
|---|---|---|
| 1 | Spherisorb CN, 5 μm, 200 × 4.6 mm | Acetonitrile + triethylamine aqueous solution (0.025 mol/L, pH = 3, adjusted by $H_3PO_4$) = 28 + 72, v + v |
| 2 | Spherigel ODS $C_{18}$, 5 μm, 250 × 4.6 mm | Acetonitrile + pic-B7 aqueous solution (5 mmol/L, pH = 3) = 50 + 50, v + v |
| 3 | Spherigel ODS $C_{18}$, 5 μm, 250 × 4.6 mm | 0.05 mol/L disodium hydrogen phosphate buffer (pH = 3, adjusted by $H_3PO_4$)+ acetonitrile = 65 + 35, v/v |

suitable for preparative separation. Table 2 lists results of the separation and sample amount loaded. From the results, different HPLC conditions give different sample sizes. Although the amount of solid phases have no significant difference, the change of mobile phase could drastically affect the sample size of preparative separation. For conditions 2 and 3, the column used was the same. The sample size under condition 3 is about 30 times of that under condition 2. Although HPLC using condition 2 is a general ion-pair RP-HPLC method for the analysis of alkaloids, and gives good resolution for the components, it had very low column capacity. The condition was not suitable for preparative separation of alkaloids. The results reported in our experiments have identified the conclusion.

Furthermore, mobiles phase composed of acetonitrile aqueous solution with different acids was used. With the modifiers that are volatile and easily removed by evaporating such as formic acid, there were serious tailing and no obvious improvement for the resolution and loading. But the resolution, loading, and tailing obtained obvious improvement with phosphoric acid and disodium hydrogen phosphate buffer. From Table 3, phosphoric acid as modifiers can improve the tailing effect more than other acids. But the mobile phase gives an unsatisfactory result when the column is injected with 0.2 mL of sample solution. With a buffer solvent system containing 0.05 mol/L disodium hydrogen phosphate buffer solution (pH = 3), there are better results. However, with the other buffer solution such as ammonia acetic and acetic acid, the loading mass is lower. So disodium hydrogen phosphate aqueous solution (pH = 3.5) was employed.

## Optimizing Mobile Phase for Preparative Separation

The key goal of mobile phase optimization is to maximize the overall productivity of the step. A semi-preparative column (300 × 7.8 mm I.D.) was





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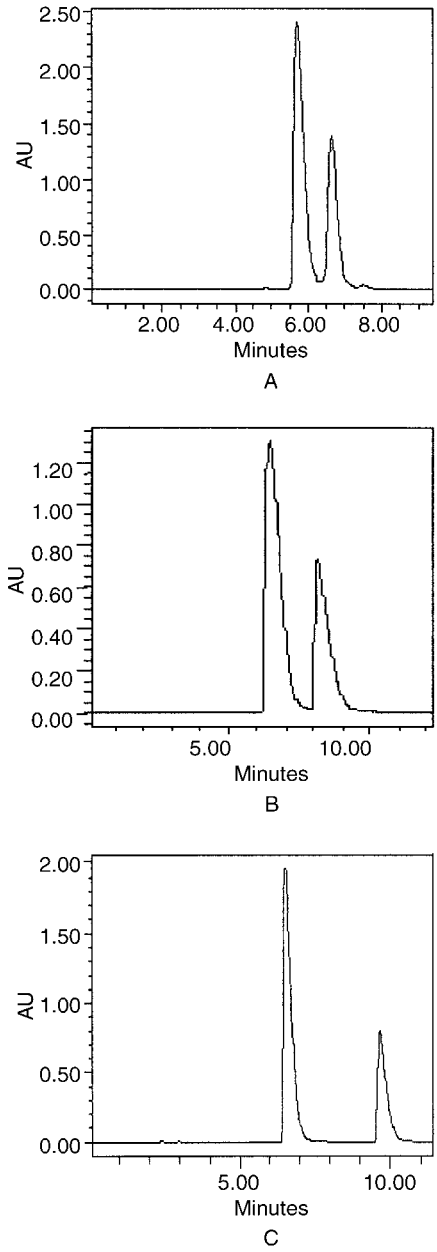


***Figure 3.*** Chromatograms of two alkaloids separation obtaining from different conditions (A, condition 1; B, condition 2; C, condition 3); UV 270 nm; injection volume: 5 μL.





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***Table 2.*** Results of resolution ($R$) with various HPLC conditions and sample size loaded.

| Injection volume[a] (mL) | $R_{(\text{condition 1})}$ | $R_{(\text{condition 2})}$ | $R_{(\text{condition 3})}$ |
|---|---|---|---|
| 0.005 | 1.4 | 1.3 | 4.0 |
| 0.010 | 1.4 | 1.3 | 4.0 |
| 0.015 | 1.3 | 1.2 | 4.0 |
| 0.025 | 1.1 | Cannot be separated | 3.5 |
| 0.100 | Cannot be separated | Cannot be separated | 2.0 |
| 0.150 | Cannot be separated | Cannot be separated | 1.9 |
| 0.200 | Cannot be separated | Cannot be separated | 1.6 |
| 0.300 | Cannot be separated | Cannot be separated | 1.4 |
| 0.400 | Cannot be separated | Cannot be separated | 1.2 |

[a]The sample solution contained 1.150 mg/mL of sanguinarine and 0.4410 mg/mL of chelerythrine.



employed to optimize separation of the alkaloids. Scaling up from analytical to semi-preparative column, the mobile phase should be optimized.

Figure 4 shows the relationship between resolution ($R$) of two alkaloids with MeCN volume fraction ($X_s$) in disodium hydrogen phosphate buffer mobile. The maximum $R$ is 1.15 when the $X$s is 0.25. Obviously, an $X_s$ of

***Table 3.*** A comparison between phosphoric acid with buffer on resolution in the analytical condition, mass, and volume of loading in the preparative condition.

| The modifiers | $R$ (analyse) | Max. load mass (mg) | | Max. load volume (mL) |
|---|---|---|---|---|
| | | Sanguinarine | Chelerythrine | |
| Formic acid (pH = 3.0) | 1.81 | 0.1150 | 0.0441 | 0.1 |
| Phosphoric acid (pH = 3.0) | 2.51 | 0.2144 | 0.1086 | 0.2 |
| 0.05 mol/L $Na_2HPO_4$ (pH = 3.0) | 4.06 | 1.150 | 0.4410 | 1.0 |
| 0.05 mol/L $NH_4AC$ (pH = 3.0) | 2.00 | 0.2144 | 0.1086 | 0.2 |

*Note:* Analytical condition: mobile phase composition: acetonitrile–water (modifiers was added, 35/65, v/v). the flow rate was 1 mL/min; inject volume: 0.5 μL; column: Spherigel ODS $C_{18}$, 5 μm, 250 × 4.6 mm. Preparative condition: mobile phase composition: acetonitrile–water (modifiers was added, 30/70, v/v) the flow rate was 2.5 mL/min; column: semipreparative column (Prep Nova-pak®HR $C_{18}$, 300 × 7.8 mm I.D.).



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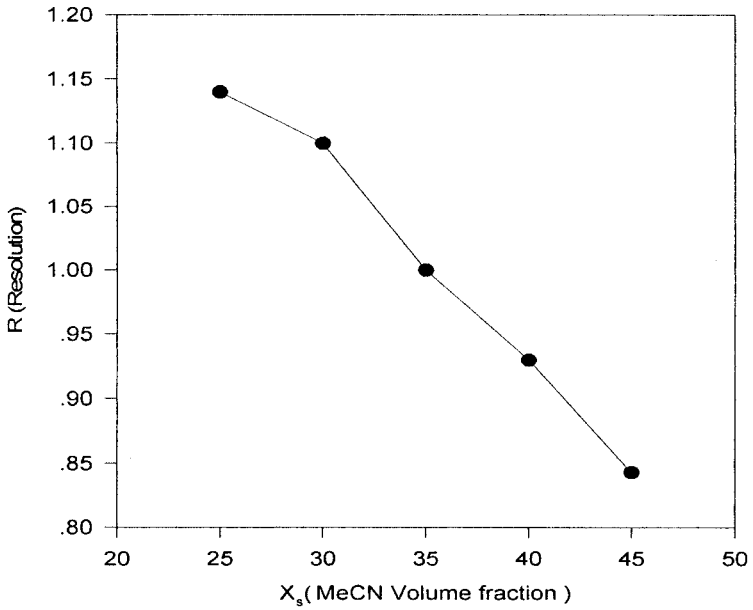


***Figure 4.*** Relationship of resolution with MeCN volume fraction in mobile phase for the separation of sanguinarine and chelerythrine. Conditions: flow rate: 2.5 mL/min; UV 270 nm, 0.5 mL of injection volume. The sample solution contained 1.150 mg/mL of sanguinarine and 0.4410 mg/mL of chelerythrine.

less than 0.25 will give a higher resolution, but the run time is too long. The mobile phase ($X_s = 0.30$) cannot only simplify the procedure appreciably, but also decrease the cycle time for preparative separation and increase the loading mass.

In the experiment, the mobile phase with different pH values was used to study the influence of pH values to resolution (Table 4). The result indicates: pH of the mobile phase ranging 5–7 could not give a good separation. The maximum $R$ is 1.2, and the pH value is 3.0. A pH value of less than 3.0 will decrease the life of the column. When pH value is at 3–3.5, the separation of two alkaloids could be also completed successfully.

In the mobile phase, the concentration of disodium hydrogen phosphate was also a key factor for preparative separation. Figure 5 shows a series of chromatograms obtaining different mobile phase containing different concentrations of disodium hydrogen phosphate conditions. On different injection volume levels (0.1 and 1 mL, respectively), the effects of the concentration

***Table 4.*** *t*, *R* for sanguinarine and chelerythrine at different pHs.

| | *t* (min) | | |
|---|---|---|---|
| pH Values | Sanguinarine | Chelerythrine | *R* (min) |
| 3.02 | 11.52 | 20.90 | 1.20 |
| 3.47 | 11.67 | 21.24 | 1.15 |
| 3.63 | 12.05 | 22.12 | 0.97 |
| 4.17 | 12.22 | 22.62 | 0.91 |
| 5.14 | — | — | Not been separated |
| 7.09 | — | — | No peak |

*Notes:* Mobile phase composition: MeCN–0.05 mol/L disodium hydrogen phosphate aqueous solution (30/70, v/v; pH = 3); flow rate: 2.5 mL/min; UV 270 nm; injected volume: 0.4 mL. The sample solution contained 1.150 mg/mL of sanguinarine and 0.4410 mg/mL of chelerythrine.



of disodium hydrogen phosphate in separations have been investigated. From the results, it can be seen that the tailing had been decreased with the increase of the concentration of disodium hydrogen phosphate. Only from the view of the separation, 100 mmol/L of disodium hydrogen phosphate gives the optimum separated efficiency on different sample sizes. However, high concentration of phosphate salt can shorten the column life, and is disadvantageous to the parts of the instrument such as pump, injector, and so on. Low concentration buffer could not restrain the tailing of the components, especially chelerythrine. So, 50 mmol/L buffer was selected.

According to the above studies, the mobile phase MeCN–0.05 mol/L disodium hydrogen phosphate buffer (pH = 3.5, adjusted by phosphoric acid) were used to separate the two alkaloids. From Table 3, the largest injected volume is 1.0 mL. Figure 5(B) (50 mmol/L $Na_2HPO_4$) shows the preparative chromatogram obtained from the chromatographic condition.

## CONCLUSION

The proposed prep-RP-HPLC can be used to purify sanguinarine and chelerythrine from the crude alkaloids of fruits of *M. cordata* (Willd) R. Br. An efficient, refined method, with the help of macroporous resin, can be used to isolate the sanguinarine and chelerythrine with the other alkaloids. The method has a number of advantages, such as high resolution and large sample size and so on. Furthermore, it has been found that the general ion



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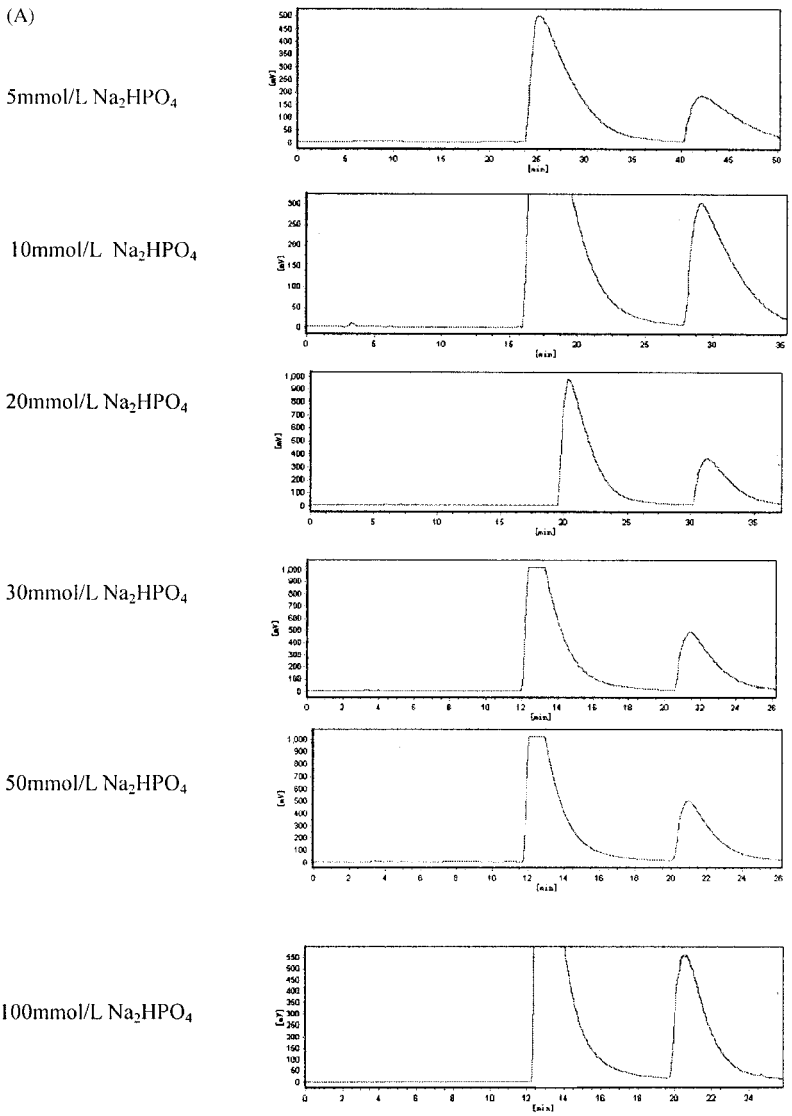


***Figure 5.*** Series of chromatograms obtained with various mobile phases containing different concentration of disodium hydrogen phosphate. Conditions: column: 300 × 7.8 mm I.D. semi-preparative column (Prep Nova-pak®HR $C_{18}$Waters); the isocratic mobile phase consisting of 0.05 mol/L sodium hydrogen phosphate buffer (pH = 3.5) and acetonitrile (70/30, v/v); flow rate: 2.5 mL/min; UV 270 nm; injected volume: 0.1 mL (A) and 1.0 mL (B). The sample solution contained 1.150 mg/mL of sanguinarine and 0.4410 mg/mL of chelerythrine.





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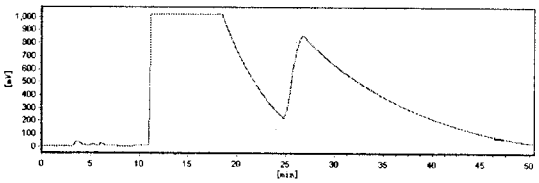
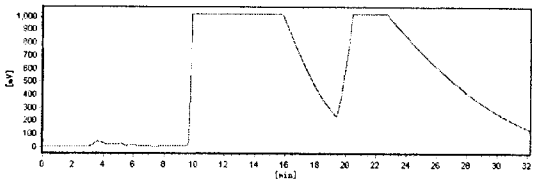
(B)

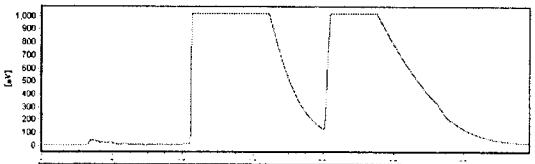
5mmol/L $Na_2HPO_4$



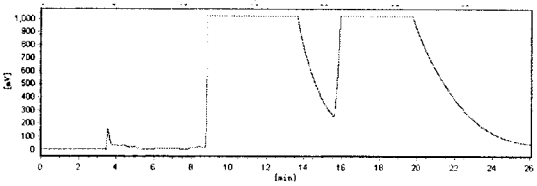
10mmol/L $Na_2HPO_4$



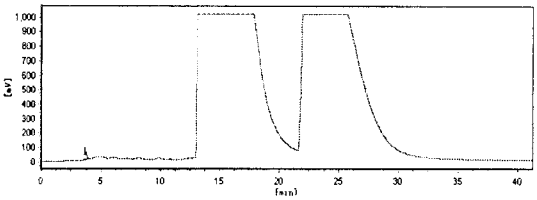
20mmol/L $Na_2HPO_4$



30mmol/L $Na_2HPO_4$



50mmol/L $Na_2HPO_4$



100mmol/L$Na_2HPO_4$

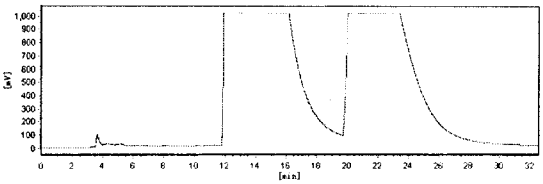


***Figure 5.*** Continued.





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pair RP-HPLC for alkaloids analysis was not suitable for preparative separation for the components because of its low sample size.

## ACKNOWLEDGMENTS

This work was supported by the Key Technologies Research and Development Program of the Tenth Five-year Plan of the Ministry of Science and Technology of the People's Republic of China and Hunan province (2001BA746C, 2001BA804A21, 2003 AA2Z3515), the Natural Science Foundation of China and Hunan province (02JJY2023).



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Received October 7, 2003
Accepted December 2, 2003
Manuscript 6241