Asian Journal of Chemistry; Vol. 24, No. 8 (2012), 3671-3674

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Analysis of *Bis*benzylisoquinoline Alkaloids by Micellar Electrokinetic Chromatography with Large Volume Sample Stacking Technology

JINHUA ZHU[1,2,*], SHUFANG TIAN[3], YANMEI ZHOU[1] and XINGGUO CHEN[2]

[1]Institute of Environmental and Analytical Sciences, College of Chemistry and Chemical Engineering, Henan University, Kaifeng, Henan 475004, P.R. China
[2]Department of Chemistry, Lanzhou University, Lanzhou 730000, P.R. China
[3]Teaching Center of Basic Experiment, Henan University, Kaifeng, Henan 475004, P.R. China

*Corresponding author: E-mail: zhujinhua0528@163.com

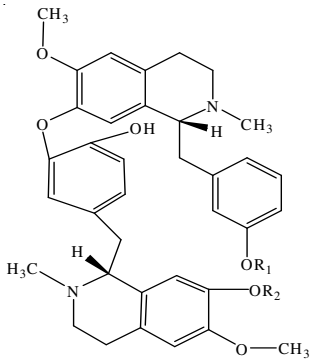
(*Received*: 5 September 2011; *Accepted*: 21 March 2012) AJC-11214

This paper presents a sensitive method for separation and quantitative analysis of three structurally similar and hydrophobic *bis*benzylisoquinoline alkaloids, liensinine, isoliensinine and neferine from embryo of the seed of *Nelumbo nucifera* Gaertn by micellar electrokinetic chromatography using a cationic surfactant, tetradecyltrimethyl ammonium bromide. In order to enhance the detection sensitivity of the analytes, large volume sample stacking technique is applied. Complete separation and stacking of the analytes is achieved within 15 min using 40 mM sodium dihydrogenphosphate (pH 6.40) containing 10 mM tetradecyltrimethyl ammonium bromide and 15 % (v/v) methanol as buffer. Detection limits in the range of 12.8-14.1 ng/mL for the analytes are obtained using large volume sample stacking. The sensitivity is improved 20-fold comparing with normal method. Finally, sample extraction is determined using this method and the result is in accordance with reference report.

Key Words: Micellar electrokinetic chromatography, Large volume sample stacking, *Bis*benzylisoquinoline alkaloids.

INTRODUCTION

Nelumbo nucifera Gaertn. (Nymphaceae) is a perennial aquatic crop grown and consumed throughout Asia, Russia and some countries in Africa. It is not only as an ornamental plant, but also for a dietary staple in eastern Asia, particularly in China. The embryo of the seed of *Nelumbo nucifera* Gaertn., a traditional Chinese drug as "Lian Zi Xin", is primarily used for treating nervous disorders, insomnia, high fevers with restlessness, cardiovascular diseases such as hypertension and arrhythmia[1-5]. Liensinine (LIE) and its analogues, isoliensinine (ISO) and neferine (NEF) (Fig. 1), are three main *bis*benzylisoquinoline alkaloids components in embryo of the seed of *Nelumbo nucifera* Gaertn[6] and they are usually applied for lotus plumule quality control. Because of their chemical and biological properties, in the past, many studies are focused on the isolation and pharmacology of these alkaloids components[7-14]. Recently, Chen et al. used micellar electrokinetic chromatography (MEKC) with a cationic surfactant and a internal standard (IS) to determine the three compounds in embryo of the seed of *Nelumbo nucifera* Gaertn[15]. The repeatability of these analytes was greatly improved with the IS method. However, the shortcoming of that method was the relatively low concentration sensitivity (the LODs in this reference is higher than that of HPLC method reported in reference[15]). Therefore, further investigation should be done for the sensitivity improvement.



No	Analogue	R_1	R_2
1	LIE	H	CH_3
2	ISO	CH_3	H
3	NEF	CH_3	CH_3

Fig. 1. Chemical structures of analytes

Although capillary electrophoresis (CE) has the advantages of high resolution capability and small sample volume

consumption, the main disadvantage of micellar electrokinetic chromatography, as it happens with other modes of capillary electrophoresis, is the low concentration sensitivity with UV detector due to the short optical path length and the small sample injection volume. To overcome this shortcoming, sample concentration steps are therefore needed to decrease the detection limits. The most simple and common used on-column preconcentration technique in capillary electrophoresis is large volume sample stacking (LVSS)[16,17]. Mechanism of large volume sample stacking is based on the difference in electrophoretic velocities between the high electric field sample zone (low-conductivity zone) and the low electric field running solution zone (high-conductivity zone). Sample ions migrate faster in the sample zone than in the running buffer solution zone and slow down when they reach the running solution zone. The analyte is focused at the boundary of the two zones. The concentration technique is based on injecting a large volume of sample dissolved in a low-conductivity solvent into the capillary. In order to preserve high resolution, the sample matrix must be pumped out of the capillary. Polarity switching and suppressed EOF are two powerful tools for sample matrix removal in capillary electrophoresis for sensitivity enhancement[18,19]. In this study, the addition of cationic surfactants to the running buffer caused the reversal of EOF. The reversed EOF directed toward the positive electrode, whereas the micelle had the electrophoretic mobility in the opposite direction[20]. Analytes were concentrated by the oppositely moved EOF and micelle without polarity switching or suppressed EOF[21,22]. After that, the analytes were brought to the detector by the EOF since the magnitude of the EOF was greater than the electrophoretic velocity of the micelle.

So, in this study, liensinine, isoliensinine and neferine in embryo of the seed of *N. nucifera* Gaertn were selected as test compounds to investigate the applicability of micellar electrokinetic chromatography-large volume sample stacking system using a cationic surfactant TTAB for the detection sensitivity improvement of the three analytes. The influence of sample injection time was investigated, the limits of detection were determined and the results were compared with that of previous work.

EXPERIMENTAL

A Waters Quanta 4000 capillary electrophoresis system (Milford, MA, USA) equipped with a UV detector was used. Data were collected and processed by a Maxima 820 chromatography workstation. The UV detection was operated at 214 nm. Electrophoresis was performed using an uncoated fused-silica capillary (Yongnian Photoconductive Fiber Factory, Hebei, China) of 55.0 cm total length (47.5 cm effective length) and 75 μm I.D. (365 μm O.D.). The temperature was maintained at 27.5 ± 0.5 ºC with a forced-air cooling system.

Chemicals and materials: Standards of liensinine, isoliensinine and neferine were purchased from Zhongshan Kangzhiyuan Biological and Scientific Company Co., (Guangzhou, China). Embryos of the seed of *Nelumbo nucifera* Gaertn were purchased from local drugstore (Lanzhou, China). TTAB was purchased from Jintan Huadong Chemical Research Institute (Jintan, China). Sodium dihydrogenphosphate (NaH_2PO_4), methanol (MeOH), sodium hydroxide and hydrochloric acid were purchased from Tianjin Chemical Reagent Factory (Tianjin, China). All chemicals and solvents were of analytical regent grade and were used without further purification. Distilled water was used throughout.

Solutions preparation: Stock standard solutions (1000 μg/mL) of liensinine, isoliensinine and neferine were prepared in 80 % aqueous MeOH. The standard solutions at various concentrations were diluted with distilled water. The standard solutions were kept in dark and stored at 4 ºC. The running buffer was a 40 mM NaH_2PO_4 solution (pH 6.40) containing 10 mM TTAB-15 % (v/v) MeOH. The buffer was prepared daily from stock solution of 0.4 M NaH_2PO_4, 0.2 M TTAB and MeOH and then adjusted to the desired pH using either 2 M NaOH or 2 M HCl. The pH values of the buffers were checked using a PHS-3B acidity meter (Shanghai Precision & Scientific Instrument Co., Shanghai, China). All the solutions were filtered through a 0.45 μm syringe filters prior to use.

Sample preparation: 1 g pulverized sample of the whole embryos of the seed of *Nelumbo nucifera* Gaertn was extracted with 80 % aqueous MeOH (10 mL) in an ultrasonic bath for 1 h, then it was filtered and fixed to the volume of 10 mL with 80 % aqueous MeOH. When large volume sample stacking/micellar electrokinetic chromatography process was performed, sample solution was diluted 20-fold with distilled water. The sample solution was kept in dark and stored at 4 ºC.

Operation procedure: Before its first use, the capillary was washed successively with 1 M NaOH for 10 min, double distilled water for 10 min, 0.1 M NaOH for 15 min and finally with water for 10 min. At the beginning of each working day, the capillary was equilibrated by rinsing with 0.1 M NaOH for 5 min, water for 5 min and then the running buffer for 5 min. The capillary was washed between consecutive analyses with water for 2 min, followed by 0.1 M NaOH for 3 min, water for 2 min and finally the running buffer for 3 min.

Samples were introduced from the cathodic end by hydrodynamic injection at 10 cm height difference. After sample injection, the sample vial was changed to the buffer solution vial and a negative voltage (-15 kV) was applied both for sample stacking and subsequent separation.

RESULTS AND DISCUSSION

In general, ionic micelles promote sweeping of analytes with opposite charges[23,24]. Experiments in this work proved that higher concentration effect was obtained with stacking rather than that of with sweeping. Therefore, a simple yet efficient stacking method-large volume sample stacking in micellar electrokinetic chromatography was investigated in this work for sensitivity improvement. The principle of large volume sample stacking/micellar electrokinetic chromatography with a cationic surfactant was the same as that with an anionic surfactant except for the using of reversed electrode polarity[25].

Method optimization: The optimization of separation conditions were as reference[15]. Finally 40 mM NaH_2PO_4 buffer solution (pH 6.40) containing 10 mM TTAB-15 % (v/v) MeOH was used. The injection time has great influence on the stacking results for large volume sample stacking method. Therefore

the injection time of large volume sample stacking was investigated in this study, using the optimum separation conditions described above. Analytes were dissolved in distilled water, the peak height was selected as evaluating index to investigate the effect of the injection time on stacking (Fig. 2). The peak height, as shown in Fig. 2, increased with an increase in the injection time up to 80 s. However, for much longer injection time (100 s), peak heights leveled off and peaks showed asymmetric and distorted shapes. One probable explanation is that when a capillary is filled with sample to a fraction greater than the amount of each analyte that can be stacked, before analytes migrate toward the anode, micelles will therefore be present in the sample matrix zone, which caused broad peaks or splitted peaks. Quirino and Terabe[26] have observed similar phenomena, but they did not give positive explanations. And also, this could be explained by the partial laminar low produced inside the capillary as a result of mismatch of EOF velocity in the sample and buffer zones when the sample zone is too long[27,28]. Finally, an injection time of 80 s was selected as the most suitable in terms of peak shapes. The optimum electropherograms of the three analytes with large volume sample stacking/micellar electrokinetic chromatography method were shown in Fig. 3A. Comparing the electropherograms obtained in reference[15] (Fig. 3A), the some difference in migration times were probably due to the time taken to remove the sample matrix from the capillary.

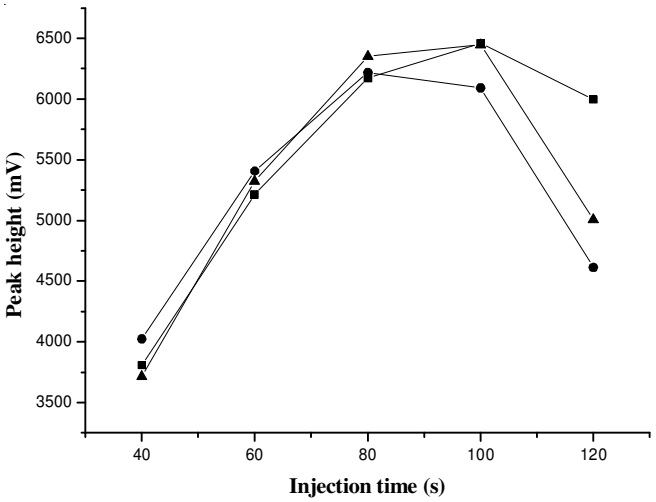


Fig. 2. Influence of injection time on peak height. Separation condition: 40 mM NaH_2PO_4-10 mM TTAB-15 % (v/v) MeOH buffer at pH 6.40, voltage, -15 kV. The concentrations of the standards were 10 μg/mL for LIE (■), NEF (●) and ISO (▲) in distilled water

Method validation: For evaluation of the quantitative applicability of the method, the *bis*benzylisoquinoline alkaloids standard solutions in the tested concentration ranges were analyzed under the optimum separation conditions. The results were shown in Table-1. As shown in Table-1, the calibration curves were found to have excellent linearity over the concentration range of 0.5-20 μg/mL with correlation coefficient (R) 0.9914-0.9929. The reproducibility of the method was determined with a standard mixture solution at the level of 10 μg/mL for all the analytes. The relative standard deviations (n = 4) of the migration times and peak areas were 2.3-3.9 % and 4.1-6.2 %, respectively. The limit of detections calculated using a S/N = 3 was in the 12.8-14.1 ng/mL range, which were much lower than that of the reported[15]. The sensitivity, evaluated by the height of the peaks, was enhanced about 19-fold in comparison with normal hydrodynamic injection. From the results it can be concluded that large volume sample stacking/micellar electrokinetic chromatography method was sensitive to improve the concentration sensitivity for the analytes using cationic surfactant. However, more sensitive stacking methods were needed to further improve the detection sensitivity.

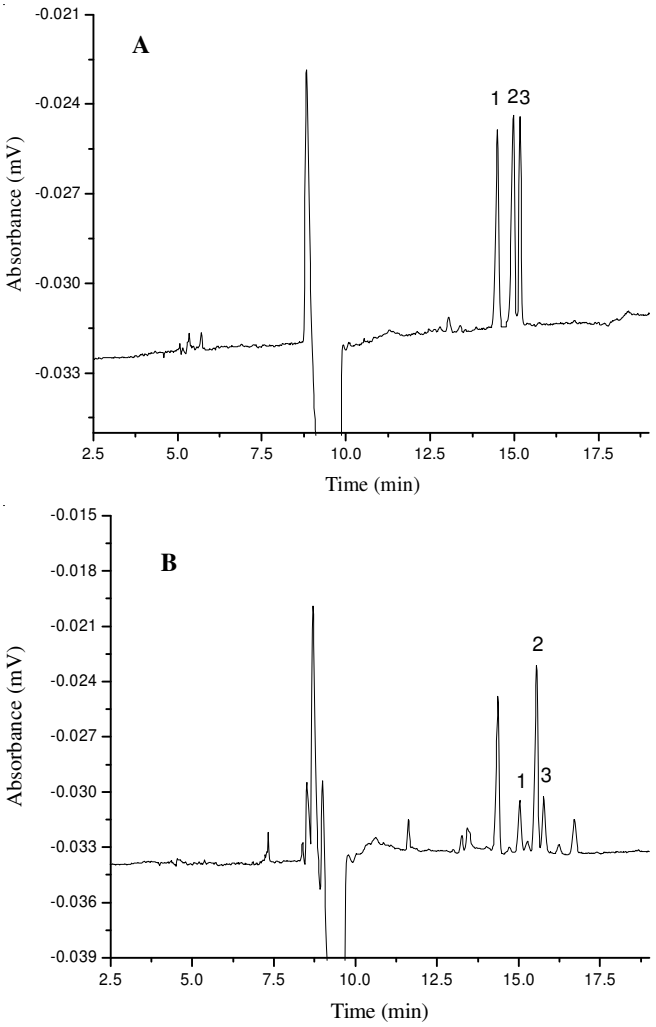


Fig. 3. Typical electropherograms of the standardsmixture solution and real samples under the optimum conditions. (A) The standards mixture; (B) The extracts of the whole embryo of the seed of N. nucifera Gaertn; the concentrations of the standards were 10 μg/mL for all the analytes, respectively; electrophoretic conditions were the same as those in Fig. 2, the injection time was 80 s. Peak recognition was as in Fig. 1

Sample analysis: All the three alkaloids extracted from the whole embryo of the seed of *Nelumbo nucifera* Gaertn were analyzed by the present method under the optimum conditions. The stock sample solution was diluted 20-fold with distilled water when analyzed. The peaks were identified by comparing the migration times and also by spiking the standards to the sample solutions. The typical electropherogram was shown in Fig. 3B. The contents of the three alkaloids found in the sample solutions mentioned above together with their

TABLE-1
LINEARITY (n=6), REPRODUCIBILITY (RSD) OF PEAK AREA AND THE MIGRATION TIMES, LODs AND STACKING ENHANCEMENT FACTORS OF LVSS/MEKC METHOD

	LIE	NEF	ISO
Regression equation[a]	$y = 3.95 \times 10^3x$-1.46×10^3	$y = 3.60 \times 10^3x$-1.43×10^3	$y = 3.57 \times 10^3x$-2.71×10^3
Correlation coefficient	0.9929	0.9914	0.9926
Linear range (μg/mL)	0.5-20	0.5-20	0.5-20
Migration time RSD (%, n = 4)[b]	2.3	3.1	3.9
Peak area RSD (%, n = 4)[b]	4.7	4.1	6.2
LOD (S/N=3) (μg/mL)	1.41×10^{-2}	1.40×10^{-2}	1.28×10^{-2}
Stacking factor[c]	19	19	18

[a]*y* and *x* were peak area (mVs) and analyte concentration (μg/mL), respectively; [b]Relative standard deviation (RSD) of intra-day of the corrected peak areas and migration times (10 μg/mL); [c]Enhancement factor = peak height obtained with LVSS procedure/peak height obtained with usual injection×dilution factor. Conditions were the same as those found in Fig. 3B

relative standard deviations (RSDs) were given in Table-2. From the results shown in Table-2, it can be seen that the contents of the three analytes were in accordance with the previous report[15]. The recoveries of the method were determined with the standard addition method for liensinine, neferine and isoliensinine in the extracts of the whole embryo of the seed of *Nelumbo nucifera* Gaertn with the result of 94-112 %.

TABLE-2
CONTENT AND RECOVERY RESULTS OF THE THREE COMPONENTS IN THE TOTAL EMBRYO OF THE SEED OF *Nelumbo nucifera* Gaertn USING LVSS/MEKC METHOD

Ingredient	Content (mg/g)	Recovery (%)	RSD (%)
LIE	1.01	94	7.3
NEF	4.55	112	5.1
ISO	1.23	106	4.6

Conclusion

In this study, a stacking method based cationic surfactant as the pseudostationary phase of micellar electrokinetic chromatography was reported and its application was discussed. The detection sensitivity was greatly improved with large volume sample stacking/micellar electrokinetic chromatography technique, the limit of detection were almost reduced two orders. The results demonstrated that the stacking technique was simple to perform, using conventional instrument without complicated procedures. The large volume sample stacking/micellar electrokinetic chromatography method reinforced the available data concerning the *bis*benzylisoquinoline alkaloids determination in embryo of the seed of *Nelumbo nucifera* Gaertn. And the results also showed that large volume sample stacking in combination with micellar electrokinetic chromatography using cationic micelles was proven to be a feasible and attractive way for improving the sensitivity of detection in capillary electrophoresis for the determination of the three analytes.

REFERENCES

1. W.Q. Lü, H.H. Zheng and X. Ge, *J. Chin. Trad. Med*., **27**, 438 (1996).
2. O. Suthienkul, O. Miyazaki, M. Chulasiri and U. Kositanont, *Southeast Asian J. Trop Med. Public Health*, **24**, 751 (1993).
3. C.P. Liu, W.J. Tsai, Y.L. Lin and J.F. Liao, *Life Sci*., **75**, 699 (2004).
4. P.K. Mukherjee, K. Saha, R. Balasubramanian, M. Pal and B.P. Saha, *J. Ethnopharmacol.,* **54**, 63 (1996).
5. C.J. Huang and M.C. Wu, *J. Biomed. Sci*., **9**, 596 (2002).
6. J.L. Wang, X.M. Hu, W.H. Yin and H.S. Cai, *China J. Chin. Mater. Med*., **16**, 673 (1991).
7. J. Yang and K. Zhou, *Magn. Reson. Chem*., **42**, 994 (2004).
8. X.M. Hu, B.H. Zhou, S.D. Luo and H.S. Cai, *J. Chin. Trad. Med*., **18**, 167 (1993).
9. X.Z. Zhang, W. Wen, X.M. Hu and S.D. Luo, *Chin. J. Chromatogr*., **15**, 347 (1997).
10. Z.Y. Li, Y. Pang and G.N. Wang, *Zhong Hua Shi Yong Zhong Xi Yi Za Zhi*, **18**, 135 (2005).
11. Y. Kashiwada, A. Aoshima, Y. Ikeshiro, Y.-P. Chen, H. Furukawa, M. Itoigawa, T. Fujioka, K. Mihashi, L.M. Cosentino, S.L. Morris-Natshke and K.-H. Lee, *Bioorgan. Med. Chem*., **13**, 443 (2005).
12. H. Zhou, H.D. Jiang, T.W. Yao and S. Zeng, *Rapid Commun. Mass Spectrom*., **21**, 2120 (2007).
13. Sh. H. Wu, C.R. Sun, X.J. Cao, H. Zhou, H. Zhang and Y.J. Pan, *J. Chromatogr. A*, **1041**, 153 (2004).
14. Y. Chen, G.R. Fan, H.L. Wu, Y.T. Wu and A. Mitchell, *J. Pharm. Biomed. Anal*., **43**, 99 (2007).
15. J.H. Zhu, H.G. Zhang, S.D. Qi, X.G. Chen and Z.D. Hu, *Chromatographia*, **73**, 535 (2011).
16. R.L. Chien and D.S. Burgi, *Anal. Chem*., **64**, 1046 (1992).
17. N. Siri, P. Riolet, C. Bayle and F. Couderc, *J. Chromatogr. B*, **793**, 151 (2003).
18. M. Albert, L. Debusschere, C. Demesmay and J.L. Rocca, *J. Chromatogr. A*, **757**, 281 (1997).
19. J.H. Lee, O.-K. Choi, H.S. Jung, K.-R. Kim and D.S. Chung, *Electrophoresis*, **21**, 930 (2000).
20. J.P. Quirino and S. Terabe, *Electrophoresis*, **21**, 355 (2000).
21. R.L. Chien and D.S. Burgi, *Anal. Chem*., **64**, 489A (1992).
22. J.P. Quirino and S. Terabe, *J. Chromatogr. A*, **850**, 339 (1999).
23. M.J. Gong, K.R. Wehmeyer, P.A. Limbach and W.R. Heineman, *Anal. Chem*., **78**, 6035 (2006).
24. J.B. Kim, J.P. Quirino, K. Otsuka and S. Terabe, *J. Chromatogr. A*, **916**, 123 (2001).
25. J.P. Quirino and S. Terabe, *J. Chromatogr. A*, **781**, 119 (1997).
26. J.P. Quirino and S. Terabe, *Anal. Chem*., **70**, 149 (1998).
27. D.S. Burgi and R.L. Chien, *Anal. Chem*., **63**, 2042 (1991).
28. R.L. Chien and J.C. Helmer, *Anal. Chem*., **63**, 1354 (1991).