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Separation, identification and rapid determination of liensine, isoliensinine and neferine from embryo of the seed of *Nelumbo nucifera* GAERTN. by liquid chromatography coupled to diode array detector and tandem mass spectrometry

Yi Chen [a,b], Guorong Fan [a,b,*], Huiling Wu [a,b], Yutian Wu [a,b], Annabel Mitchell [c]

[a] *Department of Pharmaceutical Analysis, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, PR China*
[b] *Shanghai Key Laboratory for Pharmaceutical Metabolite Research, No. 325 Guohe Road, Shanghai 200433, PR China*
[c] *Varian Australia Pty Ltd., 679 Springvale Road, Mulgrave, Vic. 3170, Australia*

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Abstract

An application of mass spectrometric methods has been developed to characterize, prepare and quantitatively analyze three bisbenzylisoquinoline alkaloids (liensinine, isoliensinine and neferine) from embryo of the seed of *Nelumbo nucifera* GAERTN. Initially, an analytical method based on liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) with positive ionization mode using a MonoChrom C18 column (4.6 mm × 250 mm i.d. 10 μm) has been developed to characterize liensinine, isoliensinine and neferine, and then scaled up to purify them on a 21.4 mm × 250 mm preparative column. The structures of liensinine, isoliensinine and neferine were elucidated by NMR. Finally, a LC–MS/MS determination method, successfully applied to separation within 3 min, was developed for high throughput simultaneous measurment of liensinine, isoliensinine, and neferine in the extract samples. Multiple reaction monitoring (MRM) was used to monitor the transition of the protonated molecules *m/z* 611, 611, 625 $[M+H]^+$ to the product ions *m/z* 206, 192, 206 for analysis of liensinine, isoliensinine and neferine. The LC–MS/MS system was linear in the concentration range of 0.0247–6.02 μg/ml with correlation coefficients of $r^2 > 0.992$. The quantitative method was validated, with an S/N = 3 detection limit of 0.15 ng for liensinine, 0.19 ng for isoliensinine and 0.12 ng for neferine. The mass fractions of liensinine, isoliensinine, and neferine in the crude extract and the phenolic alkaloid sample of embryo of the seed of *N. nucifera* GAERTN. were 16.5 ± 1.1 and 228.6 ± 11.9 for liensinine (mg/g), 45.7 ± 1.8 and 640.7 ± 15.2 for isoliensinine (mg/g), 59.7 ± 6.4 and 58.8 ± 9.8 for neferine (mg/g).
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Keywords: Preparative HPLC; LC–ESI-MS/MS; *Nelumbo nucifera* GAERTN.; Liensinine; Scale-up

1. Introduction

Nelumbo nucifera GAERTN. is a perennial aquatic crop grown and consumed over the world. It is utilized not only as an ornamental plant and a dietary staple, but also as a medicinal herb in Eastern Asia, particularly in China. Almost all parts of *N. nucifera* GAERTN., i.e., leaves, flowers, seeds and rhizomes, are utilized but rhizomes hold the largest market share. Moreover, these parts have been used for centuries in oriental medicine. The embryo of the seed of *N. nucifera* GAERTN., a traditional Chinese drug "Lian Zi Xin", is primarily used for nervous disorders, insomnia, high fevers with restlessness, cardiovascular diseases such as hypertension and arrhythmia [1–7], and also reported to show anti-HIV activity recently [8]. Liensinine and its analogues, isoliensinine and neferine (see Fig. 1), are three main bisbenzylisoquinoline alkaloids components in the embryo of the seed of *N. nucifera* GAERTN. [9]. Linesinine has been shown to slow action potentials in myocardium and slow inward current in canine cardiac Purkinje fibers [10]. Isoliensinine has exhibited a significant inhibitory effect on bleomycin-induced pulmonary fibrosis, probably due to its antioxidant and/or anti-inflammatory activities and inhibitory over expressing TNF-

* Corresponding author. Tel.: +86 21 2507 0388; fax: +86 21 2507 0388.
E-mail address: Guorfan@yahoo.com.cn (G. Fan).

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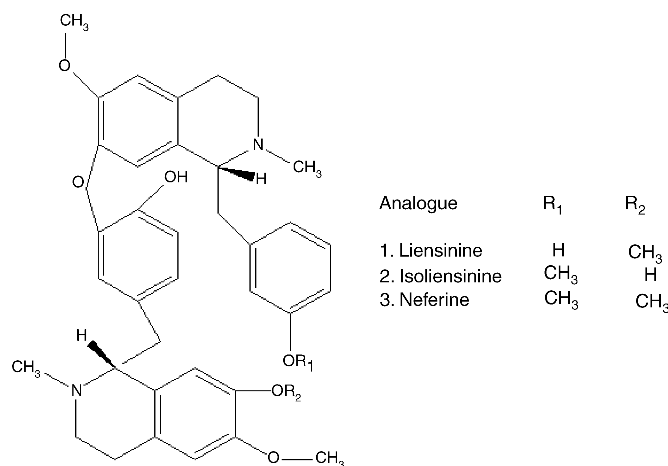

Fig. 1. Chemical structures of studied compounds.

alpha and TGF-beta(1) induced by bleomycin [11], and exerted antiproliferative effect on coronary arterial smooth muscle cells (CASMCs) induced by phenylephrine [12]. Neferine has been reported to possess a reversal effect of multidrug resistance (MDR) mediated by glutathione (GSH) detoxifcation system in K562/A02 cell line [13], reverse multidrug resistance of human gastric carcinoma SGC7901/VCR cells, which may be associated with the down-regulations of P-gp and MRP expression in SGC701/VCR cells [14], and have greater relaxant effects to rabbit cavernosal muscle stripes precontracted with phenylephine (PE), than the other extracts such as tetrandrine, kakonein, scutellarin, ginsenoside Rgl and ginsenoside Rb1 [15], and cardiovascular pharmacological effects [16].

Since the first isolation of liensinine in 1962 [17], the three alkaloids have received considerable attention because of their reputation of chemical and biological properties and many studies were focused on the isolation, quality analysis, pharmacology and pharmaceutical preparation of them [18–25]. However, their pharmacological and pharmaceutical studies often suffer from the limits of sample purity and sources. To afford pure compounds, conventional separation methods such as column chromatography (CC) and thin-layer chromatography (TLC) are very difficult and time-consuming because of their structure similarity and light sensitivity. In addition, counter-current chromatography (CCC) [26], a unique liquid–liquid partition chromatography developed recently, needs off-line high performance liquid chromatography (HPLC) detection for every peak fraction. To determine the compounds in samples, the vast majority of analysis methods, performed by using thin-layer chromatography (TLC) scanning [19,20], UV absorption [21], normal-phase and reversed-phase high performance liquid chromatography (NP/RP-HPLC) [22–25], have less specificity and sensitivity among the compounds with similar structure.

Many references [27–41] have shown that the on-line combination of LC-DAD and tandem mass spectrometric detection offers both high sensitivity and selectivity for the unambiguous identification and quantification of multiple analytes in complex samples such as herbal materials. Long sample preparation procedures can often be shortened and furthermore, this method can also be used for the quality control of the traditional Chinese medicine and to produce a chromatographic fingerprint. Although a high performance liquid chromatography-photodiode array detection-electrospray mass spectrometry method has been developed on analysis of four aporphine alkaloids from the leave of *N. nucifera* GAERTN., the contents of these compounds were simply measured by employing DAD [42]. In this paper, a high performance liquid chromatography diode array detection (DAD) coupled to positive electrospray ionization tandem mass spectrometry method was applied to the identification of liensinine and its analogues, the bisbenzylisoquinoline alkaloids in the embryo of the seed of *N. nucifera* GAERTN. for the first time, then scaled up to purify them on a 21.4 mm × 250 mm preparative column and finally a LC–MS/MS method was developed for simultaneous determination of the three alkaloids in crude extract and the phenolic alkaloid sample within 3 min.

2. Experimental

2.1. Materials

The embryo of the seed of *N. nucifera* GAERTN. was purchased from Shanghai Lei Yun Shang Pharmaceutical Co. Ltd. Hydrochloric acid, ammonia, chloroform, sodium hydroxide, ammonium acetate of analytical reagent grade were obtained from China Medicine (Group) Shanghai Chemical Reagent Corporation (Shanghai, PR. China). Acetonitrile, acetic acid and triethylamine (all chromatographic grade) were purchased from Merck (Darmstadt, German). Water was purified using a Milli-Q System from Millipore (Bedford, MA, USA).

2.2. Sample extraction

Dried and powdered embryo of the seed of *N. nucifera* GAERTN. (10 kg) was soaked in 1% HCl for 1 week. The filtrate was then precipitated with 10% aqua ammonia until the pH of the solution reached 8.5 and 593 g of crude extract were collected as brown precipitates. The crude extract was extracted three times with chloroform, and then the organic phase was extracted two times with 3% NaOH. Finally, the extract solution was precipitated with diluted HCl and off-white precipitates were filtered and dried to obtain phenolic alkaloid sample (38.5 g). The primary stock solutions of crude extract (1 mg/ml) and phenolic alkaloid sample(1 mg/ml) were prepared by dissolving appropriate amount of substance in methanol. Working solutions were obtained by diluting the stock solutions with distilled water and 10 μl acetic acid per ml solution was added. All the solutions were stored at 4 °C.

The phenolic alkaloid solution (1 mg/ml) was injected to analytical LC–ESI-MS/MS to identify the containing alkaloids. And then the analytical LC conditions were linearly scaled up to afford target alkaloids on the preparative LC. Fractions were collected after LC separation. The lyophilized powder of every fraction by Christ Alpha 1–2 Freeze Dryer (German) was obtained following evaporation of organic solvent. LC–DAD and ESI-MS/MS were used for preliminary product identifica-

tion. The products were finally verified by Mass spectrum and ^{1}H- and ^{13}C NMR.

2.3. LC–ESI-MS/MS

Identification and quantification of the analytes were carried out using a Varian LC–MS/MS system (Palo Alto, CA, USA) equipped with a ProStar 410 autosampler, two ProStar 210 pumps, a 335 diode array detector and a 1200 L electrospray tandem mass spectrometer. Firstly, a solution of phenolic alkaloid sample (10 μg/ml) was infused into the electrospray source at a constant flow-rate of 30 μl/min to tune the mass spectrometer parameters, using a Harvard Model 11 Plus syringe pump. Then, the analysis was carried out using a MonoChrom C18 column (4.6 mm × 200 mm i.d., 10 μm, Varian Inc., USA) and the mobile phase of acetonitrile-water (containing 0.2% acetic acid (v/v) and 0.1% triethylamine (v/v)) (16:84, v/v) at a flow rate of 1.4 ml/min (the column effluent was split: 0.2 ml/min to MS and 1.2 ml/min to DAD) with the column temperature kept at 30 °C and the injection volume of 20 μl. The DAD recorded UV spectra in the range from 190–400 nm with HPLC chromatogram monitored at 282 nm. The electrospray capillary potential was set to 55 V. Nitrogen was used as a drying gas for solvent evaporation. The API housing and drying gas temperatures were kept at 50 and 300 °C. The spectra over the mass range of *m/z* 350–800 spectra of the phenolic alkaloid solution was collected with the scan time 1 s and the detector multiplier voltage 1090 V.

For lab-scale preparative chromatography experiments, a Varian prep-HPLC system consisting of a manual injector with a 10-ml loop, two PrepStar 218 pumps, a 325 UV–vis dual wavelength detector and a 701 Fraction Collector was used. After filling the loop of the manual injector with 4 ml sample, the analysis was carried out using a MonoChrom C18 column (21.4 mm × 200 mm i.d., 10 μm, Varian Inc., USA) at a flow rate of 30 ml/min with the column temperature kept at 30 °C and UV detector recorded at 282 nm. The overall run time of the method was set at 30 min.

An Agilent Zorbax Elipse XDB-C18 column (3.0 mm × 100 mm i.d., 3.5 μm, Agilent Technologies, USA) was used for quantitative determination with the mobile phase consisting of acetonitrile- 0.01 M ammonium acetate (70:30, v/v) under isocratic conditions at a flow rate of 0.4 ml/min with the injection volume of 20 μl and the total analysis time of 3 min.

2.4. Validation of the quantitative analysis

The alkaloids were weighed, dissolved in methanol, degassed in an ultrasonic bath and filtered through a 0.45 μm membrane filter. Then they were diluted with water and added with 10 μl acetic acid per ml in a volumetric flask to obtain standard solutions for the calibration curves.

Calibration was performed by the internal standard. The internal standard (tetrahydropalmatine) solution was prepared by diluting substance with methanol. Each sample contained 25 ng/ml of internal standard. The ranges of calibration curves were 0.0294–2.86 μg/ml for liensinine, 0.0373–6.02 μg/ml for isoliensinine, and 0.0247–0.533 μg/ml for neferine. For each calibration curve, five different concentrations were used. The contents of them in the quality control (QC) samples including the crude extract and processed sample (the phenolic alkaloids) were calculated with the respective calibration curves.

The following criteria were used to evaluate the method: sensitivity, linearity (r^2), repeatability, accuracy and stability. Sensitivity was assessed by evaluating the LOD and LOQ values. Measurement of intra- and inter-day variability was utilized to determine the repeatability of the method. The intra- and inter-assay precision was determined by the RSD obtained on one day and on different days at three levels. Accuracy experiments were carried out using standard addition method. The recovery of the added standard alkaloids was studied by spiking the phenolic alkaloid sample with the standard solution at three different mass concentrations. Triplicate experiments at each level were performed. The recoveries for the three alkaloids were calculated by subtracting the mass concentrations of non-spiked herb extracts using internal standard linear regression. The accuracy was expressed as the percentage of alkaloids recovered by the assay. Since the three alkaloids were relatively stable when stored at 4 °C [43], the stability was tested with the standard solution of three different concentrations that were stored at room temperature and analyzed every 2 h within 12 h.

3. Results and discussion

3.1. Optimization of LC–ESI-MS/MS parameters

To obtain better sensitivity and detection, the ESI parameters were optimized in a flow injection sequence. Due to the basic nature of the alkaloids, protonation was found to be a much more effective ionization mode than deprotonation. Temperature and pressure of nebulizer gas were checked in the range of 150 °C 19 psi to 350 °C 21 psi and the maximum response found at 300 °C 21 psi. Other parameters such as the needle, shield and capillary voltage were tuned automatically with the selected ion at *m/z* 611, the most abundant ion in the full-scan spectrum. The optimum parameters for the needle was 5400 V, shield 600 V and capillary voltage 55 V. Based on the MS and MS/MS spectrum, the further study of LC–MS/MS was set to monitor the transitions of the precursors to the product ions, as follows: *m/z* 611 > 150–620 and *m/z* 625 > 150–630.

As a report indicates [25], the mobile phase of acetonitrile–water (containing 0.1% triethylamine and adjusted to pH 3.3 with phosphoric acid) was utilized to analyze the contents of phenolic alkaloids in embryo of the seed of *N. nucifera* GAERTN. On the base of the analysis conditions, the mobile phase of acetonitrile-water (containing 0.2% acetic acid and 0.1% triethylamine) and acetonitrile–0.01 M ammonium acetate were chosen for chromatographic separations because these buffers are quite compatible with LC–MS. Different proportions of acetonitrile in the mobile phase were used. Under isocratic elution, the mobile phase of acetonitrile–water (containing 0.2% acetic acid and 0.1% triethylamine) (16:84, v/v) was used to achieve better separation. LC–UV chromatograms of the phenolic alkaloid sample with their daughter scan LC–MS/MS spectra have been showed in Fig. 2. Three alkaloids were identified to be liensi-

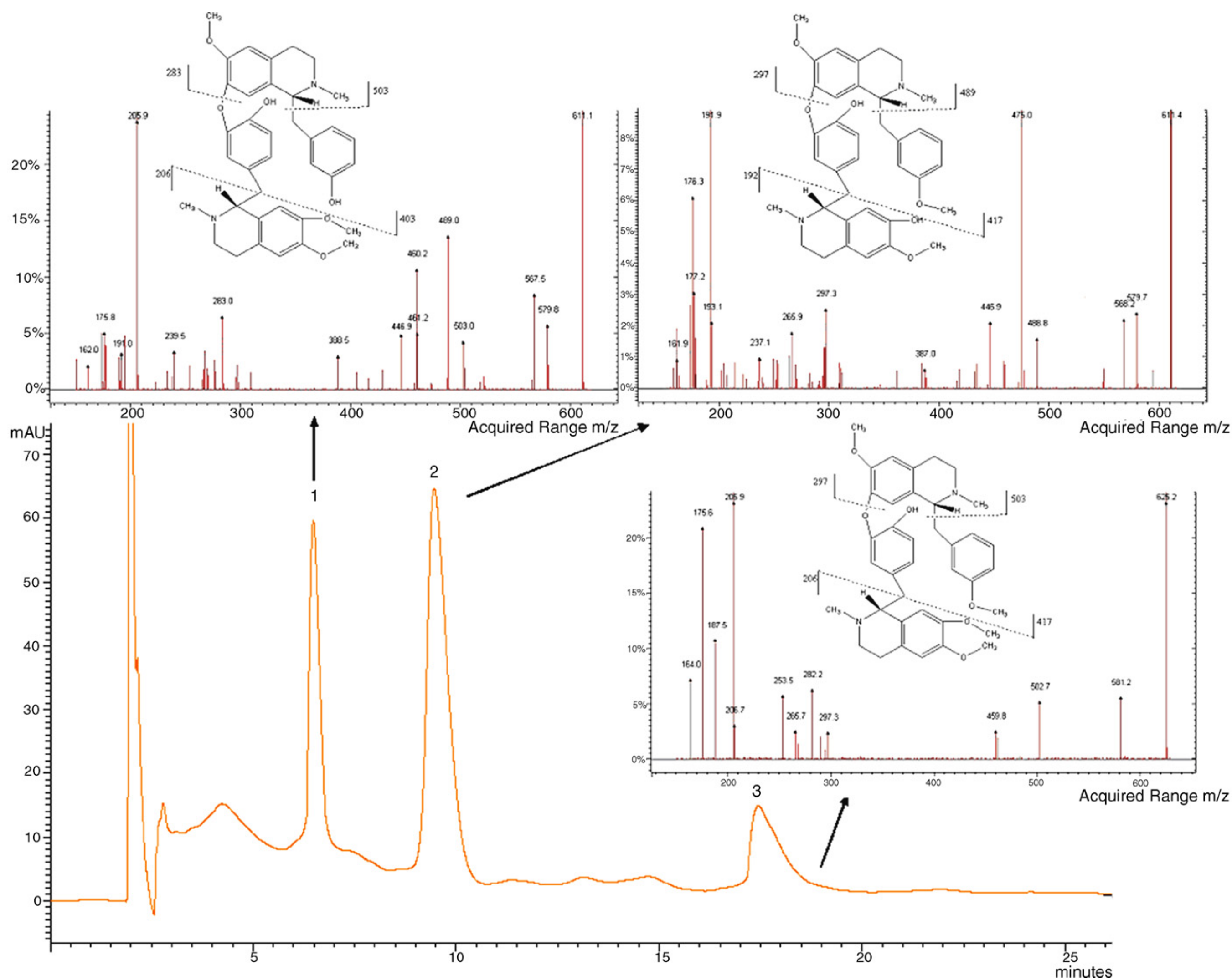

Fig. 2. Analytical LC chromatogram of the phenolic alkaloid sample at 282 nm with their daughter scan LC–MS/MS spectra. (1) liensinine, (2) isoliensinine, and (3) neferine.

nine (t_R = 6.609 min), isoliensinine (t_R = 9.472 min) and neferine (t_R = 17.716 min) using the observed UV λ_{max} (282 nm), the $[M+H]^+$ (ions at m/z 611 attributed to liensinine and isoliensinine, ion at m/z 625 attributed to neferine), the MS/MS fragments with structure analysis and the published characteristics [25,26]. By the optimum cone voltage setting at 24 eV, the most notable fragments (ions at m/z 206 attributed to liensinine and neferine, ion at m/z 192 attributed to isoliensinine) were produced. Moreover, the precusor ions at m/z 611, 611, 625 exhibited characteristic fragments at m/z 503, 283, 403, 489, 297, 417 which are illustrated in the structural cleavage of the three alkaloids in Fig. 2. Further fragments were yield, such as 580, 568, 460, 447, 475, 176, 282, 254, 188, 164, signaling loss of CH_2, CH_3, OCH_2 or CH_3CO from the parent ions or the major fragments. The loss of groups was observed in no sequence.

3.2. preparation and identification of isolated compounds

A MonoChrom C18 column (21.4 mm × 250 mm i.d., 10 μm) was used to prepare the relative large amount of the three alkaloids from the phenolic alkaloid sample. The preparative LC chromatogram was similar to the analytical LC chromatogram as in Fig. 2. Ten times of preparation were repeated. Three fractions of every preparation were combined respectively. Following evaporation of organic solvent, 9.0 mg of peak one, 25.3 mg of peak two, and 2.1 mg of peak three were obtained after lyophilization. The final verification by NMR was achieved comparing with the literature data [17,18,26]. The purity of the three alkaloids were measured by HPLC-DAD with the results of 98.1%, 96.2% and 95.5%, respectively.

3.3. Validation results

Internal calibration was used for quantitative determination of liensinine (t_R = 1.525 min), isoliensinine (t_R = 1.721 min) and neferine (t_R = 1.975 min) in the ESI-MS/MS MRM mode of precursor $[M+H]^+$ ions at m/z 611, 611, 625 into product ions at m/z 206, 192, 206 in the extract samples, with internal standard tetrahydropalmatine (t_R = 2.143 min) at m/z 356 > 192. The area ratio of each analyte to I.S. was calculated. The

Table 1
LODs, LOQs and calibration characteristics

Alkaloid	LOD (ng)	LOQ (ng)	Linearity range (μg/ml)	Regression model	r^2
Liensinine	0.15	0.59	0.0294–2.86	$Y = 0.2089 + 1.151x$	0.994
Isoliensinine	0.19	0.75	0.0373–6.02	$Y = 0.008082 + 1.686x$	0.992
Neferine	0.12	0.49	0.0247–0.533	$Y = -0.04685+1.152x$	0.997

Table 2
Precision and recovery data

Alkaloid	Intrarun precision ($n = 3$, %R.S.D.)			Interrun precision ($n = 3$, %R.S.D.)			Recovery ($n = 3$, %)					
	Low	Medium	High	Low	Medium	High	Low	R.S.D.	Medium	R.S.D.	High	R.S.D.
Liensinine	6.1	2.4	1.8	7.8	5.9	2.7	96.8	3.3	102.5	2.1	106.7	3.4
Isoliensinine	10.3	1.7	2.1	7.1	2.0	3.9	96.1	4.5	99.4	2.7	97.0	3.1
Neferine	8.9	3.6	1.9	12.7	1.8	2.3	95.2	6.1	110.9	6.3	94.1	4.8

LC–ESI-MS/MS system was linear in the concentration range of 0.0247–6.02 μg/ml with correlation coefficients of $r^2 > 0.992$.

Using a signal-to-noise ratio of 3 and 10, respectively, the LOD for liensinine, isoliensinine and neferine was 0.15, 0.19 and 0.12 ng while LOQ values were 0.59, 0.75 and 0.49 ng, respectively. The calibration curve characteristics for each alkaloid are listed in Table 1.

The precision for intra- and inter-assay runs were evaluated using standard solution prepared at three levels (LQC contained 0.0294, 0.0373 and 0.0247 μg/ml of liensinine, isoliensinine and neferine, respectively; MQC contained 0.178, 0.377 and 0.133 μg/ml of liensinine, isoliensinine and neferine, respectively; HQC contained 2.86, 6.02 and 0.533 μg/ml of liensinine, isoliensinine and neferine, respectively). The phenolic alkaloid sample was spiked the standard solution at three different mass concentrations (LQC and MQC were the same as precision evaluation while HQC contained 1.43, 3.01 and 0.267 μg/ml of liensinine, isoliensinine and neferine, respectively) to evaluate the recovery. The intra- and inter-assay precision and recovery results are shown in Table 2. The stability was tested at the same concentration levels of those in intra- and inter-day precision study and the analytes were found to be rather stable within 12 h (RSD < 3%).

The contents of the individual alkaloids in crude extract and phenolic alkaloid sample were determined by the method established above. The mass fractions of liensinine, isoliensinine and neferine in the crude extract and the phenolic alkaloid sample of embryo of the seed of *N. nucifera* GAERTN. were 16.5 ± 1.1 and 228.6 ± 11.9 for liensinine (mg/g), 45.7 ± 1.8 and 640.7 ± 15.2 for isoliensinine (mg/g), 59.7 ± 6.4 and 58.8 ± 9.8 for neferine (mg/g). The results show the majority of liensinine and isoliensinine was held in the phenolic alkaloid sample and almost all neferine was left in the chloroform organic phase in the extract procedure. It provides us a simple method to separate neferine from liensinine and isoliensinine without using time and organic solvent-consuming column chromatography.

The contents of liensinine, isoliensinine and neferine in phenolic alkaloid sample were somehow lower or higher than those determined using HPLC–UV in our previous literature [25]. Compared with HPLC–UV techniques, determination of the alkaloids in samples using LC–MS/MS has the advantages of much higher selectivity and sensitivity utilizing the characteristic reaction monitoring for each alkaloid. Therefore, this technique can provide satisfactory resolution of the peaks and eliminate the interference even with a shorten elution time of these alkaloids on the HPLC column. Accurate quantitative analysis does not solely rely on the HPLC baseline separation which requires sophisticated optimization of chromatographic conditions. The simultaneous quantification of three alkaloids in samples using LC–MS/MS method can be achieved in three minutes.

4. Conclusions

This paper described a novel method for preparation and a simple method for rapid determination of liensinine, isoliensinine and neferine in crude extract and the phenolic alkaloid sample from embryo of the seed of *N. nucifera* GAERTN. The three alkaloids were separated and identified simultaneously with HPLC–DAD-ESI-MS/MS and obtained with good purity by the scale-up of preparative LC. The structures of them were finally verified by NMR techniques. Compared with other methods, LC–MS/MS improved the specificity and sensitivity, shortened the analytical time of the samples in quantitative analysis. From this study, an appropriate example was made for identification of unknown compounds by analytical HPLC–DAD-ESI-MS/MS which provides preliminary information of structures, and then scaled up on the preparative LC to afford purified substance and final verification of the separates can be achieved by NMR techniques. Furthermore, a rapid quantitative analysis method of LC–MS/MS was also developed for high through-put determination of the compounds in crude and processed samples in quality control of traditional Chinese medicine (TCM).

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