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Rapid quantification of four major bioactive alkaloids in *Corydalis decumbens* (Thunb.) Pers. by pressurised liquid extraction combined with liquid chromatography-triple quadrupole linear ion trap mass spectrometry

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ABSTRACT

A new method based on pressurised liquid extraction (PLE) followed by liquid chromatography–triple quadrupole linear ion trap mass spectrometry (LC–QTrap-MS) analysis has been developed for the identification and quantification of four major alkaloids in extracts of *Corydalis decumbens* (Thunb.) Pers. PLE extractions were performed using 90% ethanol; temperature was set at 100 °C and pressure at 1500 psi. HPLC analysis was performed on a Waters XBridge™ C_{18} column (150 mm × 2.1 mm i.d., 3.5 μm) eluted by a mobile phase of acetonitrile and 0.2% acetic acid. Data acquisition was carried out in multiple reaction monitoring transitions (MRMs) mode, monitoring two MRM transitions to ensure an accurate identification of target compounds in the samples. Additional identification and confirmation of target compounds were performed using the enhanced product ion modus (EPI) of the linear ion trap. The novel LC–QTrap-MS platform offers the best sensitivity and specificity for characterization and quantitative determination of the four alkaloids in *C. decumbens* (Thunb.) Pers. and fulfils the quality criteria for routine laboratory application.

1. Introduction

Traditional Chinese medicine (TCM) has been attracting more and more attention in recent years because of its complementary therapeutic effects to western medicines, and its capability to deal with many essential problems that have not yet been solved by conventional medicinal practices. The rhizome of *Corydalis decumbens* (Thunb.) Pers. (family Papaveraceae) is one of the popular TCM with a Chinese name "Xiatianwu" and is officially listed in the Chinese Pharmacopoeia [1]. It has been widely used for the treatment of hemiplegia, sciatica and rheumatic arthritis [2]. Recently, animal experiments showed that its alkaloids can inhibit platelet aggregation [3–5], facilitate the protection against cerebral ischemia/reperfusion damage [6] and ameliorate the learning and memory deficit of Alzheimer's disease (AD) model rats [7]. The major active constituents of this herb are considered to be alkaloids, including bicuculline, protopine, tetrahydropalmatine, palmatine hydrochloride, whose chemical structures are given in Fig. 1A–D [8]. Bicuculline, protopine, tetrahydropalmatine, palmatine hydrochloride have been found to be effective on alleviating pain, and the efficiency decreases in order [9–12]. Therefore, their rapid and accurate identification is of great significance in the quality control of this natural medicine and its formulations.

There have been several literatures about the analysis of these alkaloids in *Corydalis yanhusuo*, but they mainly focused on the high performance liquid chromatography (HPLC) analysis [13–15], and only very few focused on mass spectrometric analysis [16–18].

Due to the unique selectivity and sensitivity, liquid chromatography–tandem mass spectrometry (LC–MS/MS) with a triple quadrupole in multiple reaction monitoring (MRM) mode has become the most widely used technique for the quantification of active constituents in TCM as reported extensively in the literatures [19–25].

Pressurised liquid extraction (PLE) generally operates under high pressure, and at higher temperature above the boiling point of the extraction solvents. High temperature enhances extraction efficiency because it decreases the viscosity of the solvent, thus allowing better penetration of solvent molecules into the sample matrix [26]. The use of PLE decreases the total extraction time, and improves extraction efficiency through the manipulation of parameters such as temperature, time, cycles and solvent [27–33].

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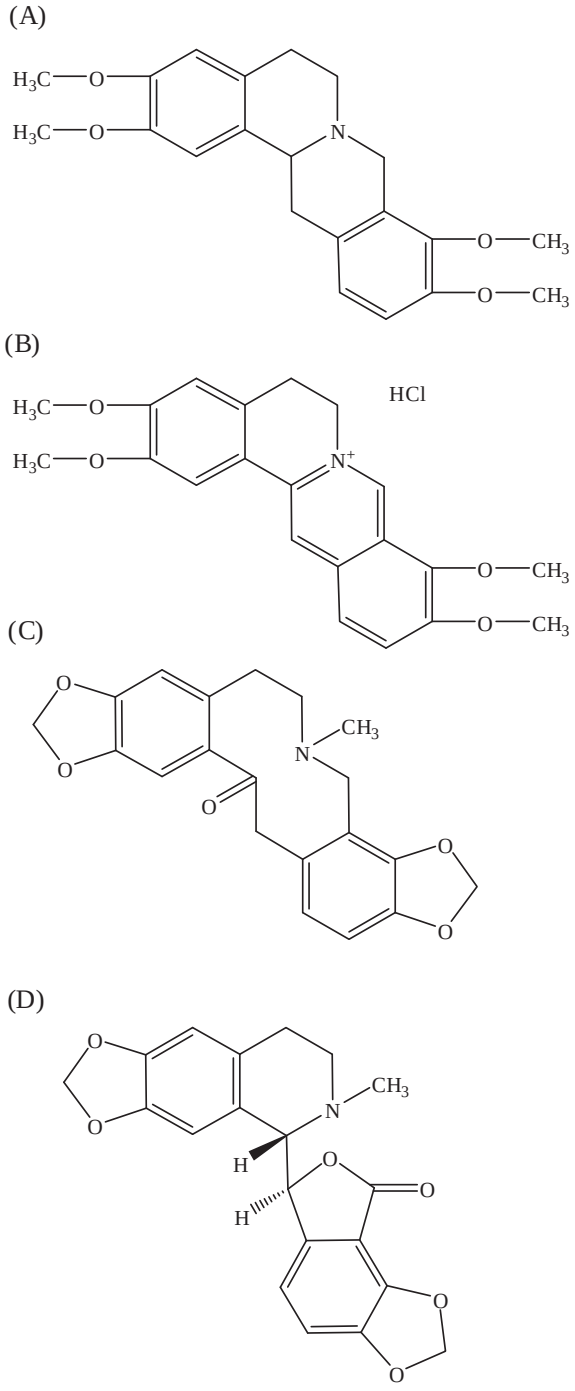

Fig. 1. Chemical structure of tetrahydropalmatine (A), palmatine hydrochloride (B), protopine (C) and bicuculline (D).

The aim of this work was to develop and to evaluate a reliable faster and less sample consuming method for the rapid simultaneous quantification of four major alkaloids in extracts of *C. decumbens* (Thunb.) Pers. using PLE extraction combined with LC–MS/MS and simultaneous identification and confirmation them by the characteristic fragment patterns obtained by the linear ion trap (QTrap) function.

2. Experimental

2.1. Reagents and standards

HPLC grade acetonitrile, methanol and acetic acid (purity 100%, w/w) were obtained from Merck (Darmstadt, Germany). The water used was purified with a Milli-Q water purification system from Millipore (Bedford, MA, USA).

All reagents were of analytical-reagent grade unless mentioned otherwise. Standards of tetrahydropalmatine, palmatine hydrochloride, protopine and bicuculline were obtained from Shanghai Standard Biotech Co., Ltd of China.

Single tetrahydropalmatine, palmatine hydrochloride, protopine and bicuculline standard solution (100 mg L^{-1}) were prepared in methanol and stored at 4 °C in the dark. Mixed calibration standards were prepared at concentrations of 1.0, 2.0, 5.0, 10.0 and 20.0 μg L^{-1} before use.

2.2. Sample preparations

Samples of *C. decumbens* (Thunb.) Pers. were purchased from different medical halls in Wenzhou and Hangzhou of China and stored at room temperature until analysis. Dried *C. decumbens* (Thunb.) Pers. samples were milled to powder (ca. 60 mesh) by a disintegrator made in Hangzhou Chunjiang Pharmacy Machine Co.

2.3. Extraction

2.3.1. Ultrasonication-assisted extraction

Ultrasonication-assisted extraction (UAE) was carried out by mixing 1.0 g of dried finely powdered sample and 50 mL of 90% ethanol in a flask, which was then placed in an ultrasonic bath for 1 h. The extraction was repeated one time and the extracts were combined. The combined extract was filtered, and the filtrate was evaporated to dryness using a rotary evaporator at 50 °C. Then, the residue was dissolved with 50 mL of methanol. The methanol extract was diluted 1/5000 with methanol and filtered through a 0.45 μm nylon filter membrane before analysis.

2.3.2. Soxhlet extraction

Soxhlet extraction was performed in a BUCHI Extraction System B-811 (BUCHI, Switzerland). Soxhlet extraction of the sample (1.0 g) was performed with 50 mL of 90% ethanol for 2 h. The extraction was repeated one time and the extracts were combined. The extract was treated in the same way as that obtained by UAE.

2.3.3. Microwave-assisted extraction

The ETHOS 1 laboratory microwave system (Milestone, Leutkirch, Germany) equipped with a 12-vessel carousel operated in the closed-vessel mode and featuring a magnetic stirring device was used for the microwave-assisted extraction (MAE) step. PTFE-lined extraction vessels and a fiber optic temperature sensor in the interior of the microwave oven were used, and both temperature and pressure were monitored during operation. Powdered sample (1.0 g) was transferred into microwave extraction vessels and suspended in 25 mL of 90% ethanol. According to a pre-designed experimental trial: 0–5 min, temperature rise to 80 °C; then temperature was kept constant at 80 °C for 25 min. After extraction, the vessels were cooled down to room temperature before opening. The extraction was repeated one time and the extracts were combined. The extract was treated in the same way as that obtained by UAE.

2.3.4. Pressurised liquid extraction

Pressurised liquid extraction (PLE) was performed using a Dionex PLE 350 Extractor (Dionex, Sunnyvale, CA, USA). About 1.0 g of dried finely powdered sample was mixed with laboratory sand and loaded into Dionex standard 22 mL stainless steel extraction cells containing Dionex standard 22 mL cellulose extraction thimbles. Extractions were performed using 90% ethanol; temperature was set at 100 °C and pressure at 1500 psi. Two different extraction steps were performed consecutively on the same sample cell. Each step consisted of an initial cell heat-up time of 10 min, followed by

Table 1
Optimized MS/MS parameters for the determination of 4 alkaloids.

Compounds	Rt (min)	MRM parameters		
		MRM transitions (m/z)	DP	CE
Bicuculline	4.81	368.1–307.1*	75	32
		368.1–190.3	75	32
Protopine	6.66	354.1–189.2*	75	39
		354.1–149.2	75	38
Tetrahydropalmatine	7.72	356.2–192.3*	90	39
		356.2–165.3	90	35
Palmatine hydrochloride	9.43	352.2–336.4*	90	40
		352.2–308.4	90	38
Mass spectrometry		Optimized value		
Ionization		ESI positive mode		
Source temperature, (°C)		500		
Ionization voltage (V)		5000		
Ion source (GS1) setting		50		
Ion source (GS2) setting		40		
Curtain gas setting		15		
CAD gas setting		10		
Dwell time		100 ms		

* MS/MS transition used for quantification.

two static 3-min cycles. After each cycle, the cell was flushed with solvent (60% of cell volume) and purged with nitrogen for 60 s. The extracts of the different extraction steps were collected and treated in the same way as that obtained by UAE.

2.4. Liquid chromatography

An Agilent 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) consisting of a solvent degassing unit, a quaternary pump, an autosampler and a thermostatted column compartment was used in the LC–MS/MS system. Separation of the analytes was achieved on a Waters XBridge TM C_{18} column (150 mm × 2.1 mm i.d., 3.5 μm) with a column oven temperature of 30 °C. The mobile phase consisted of acetonitrile (A)/0.2% acetic acid (B). The flow rate was set at 0.25 mL min^{-1}. The gradient elution program started at an initial composition of 20:80 A/B (v/v). Gradient elution employed with the ratio of A:B varied as follows: 0 min, 20:80; 10 min, 30: 70; 10.1 min, 20:80; 18 min, 20:80. Flow was diverted to waste from 0 to 3.5 min and from 11 to 18 min.

2.5. Mass spectrometry

A triple quadrupole mass spectrometer with trap function (API 4000 QTrap, Applied Biosystems, SCIEX Toronto, Canada) with a Turbo Ion Spray interface was used. Ionization was achieved using ESI in the positive mode at 500 °C with N_2 as the nebulizer. Detection was performed in multiple reaction monitoring (MRM) mode of selected ions at the first (Q1) and third quadrupole (Q3). To choose the fragmentation patterns of m/z (Q1) → m/z (Q3) for the analytes in MRM mode, direct infusion into the MS of single standard solution in methanol was performed and the product ion scan mass spectra were recorded. Once the fragment ions were chosen, the MRM conditions were further optimized for the analytes to obtain maximum sensitivity for the compound of interest (Table 1). Fragment spectra used for analytes confirmation were acquired with the enhanced product ion modus (EPI) of the linear ion trap.

3. Results and discussion

3.1. Optimization of MS parameters and MRM transitions

Preliminary experiments were conducted with the purpose of finding the best instrumental conditions that would allow unambiguous identification of the analytes in real samples at trace levels. Single tetrahydropalmatine, palmatine hydrochloride, protopine and bicuculline standard solution (100 ng μL^{-1}), prepared in methanol, and were introduced into the MS at a flow rate of 5 μL min^{-1} using a syringe pump (Harvard Apparatus, Australia).

Identification of the parent ion and the choice of the ionization mode for four alkaloids were performed in the full-scan mode by recording mass spectra from m/z 50 to 500 in positive mode. The most sensitive transition in MRM mode was selected for quantification in the screening method. A minimum of three identification points are required to meet the identification performance criteria defined by the EU Commission for quantitative mass spectrometric detection [34]. Using LC–MS/MS to monitor one precursor ion and two daughter ions 'earns' four identification points (1 for the parent ion and 1.5 for each daughter ion) and therefore fulfils these criteria.

To choose the transitions in the MRM mode, different parameters were studied. The precursor and the product ions of tetrahydropalmatine, palmatine hydrochloride, protopine and bicuculline were selected. The last parameter optimized was the collision energy and declustering potential; different values were tested (20–60 V). In Table 1, we summarize the optimum values for each condition for the four alkaloids compounds. The optimization was done following the normal optimization procedure. In this work, the most intensest characteristic MRM transitions were chosen for the four alkaloids standards, and Table 1 lists the precursor and daughter ions monitored.

3.2. Optimization of chromatographic conditions

To improve alkaloids chromatographic separation, different columns (ZORBAX Eclipse Plus C_{18} 2.1 mm × 150 mm, 3.5 μm; ZORBAX Eclipse Plus C_8 2.1 mm × 150 mm, 3.5 μm; Waters Symmetry column C_{18} 2.1 mm × 150 mm, 3.5 μm; Waters X Bridge™ C_{18} 2.1 mm × 150 mm, 3.5 μm) and mobile phases were investigated in this work. The results indicated that for the columns investigated, the Waters XBridge C_{18} column was the optimal one. To enhance the signal response, mobile phase modifiers such as ammonium acetate, acetic acid and formic acid were also investigated. 0.2% acetic acid was found to provide the maximum response and was used in the following method development. The final gradient composition is composed of acetonitrile and 0.2% acetic acid. As shown in Fig. 2, the four alkaloids were adequately separated from each other. The results also suggest that the sufficient baseline chromatographic separation has the benefit of reducing the matrix effect.

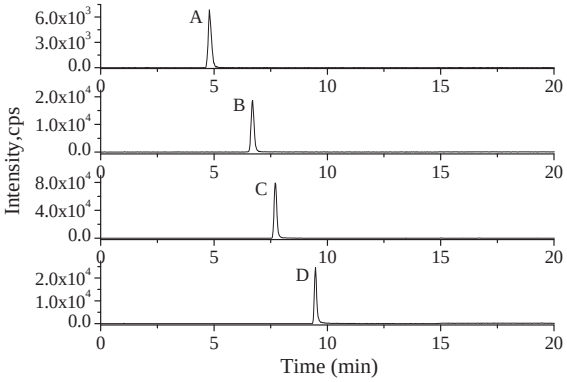

Fig. 2. LC–MS/MS MRM chromatogram of a *Corydalis decumbens* (Thunb.) Pers. sample. Bicuculline (A), protopine (B), tetrahydropalmatine (C), palmatine hydrochloride (D).

Table 2
Factors in the orthogonal design for the optimization of extraction conditions.

Run no.	A: ethanol content in solvent (%)	B: extraction time (min)	C: extraction temperature (°C)	D: Sample amount (g)	T^a (%)
1	50	5	80	0.5	0.29
2	50	10	100	1.0	0.34
3	50	20	120	1.5	0.32
4	70	5	100	1.5	0.35
5	70	10	120	0.5	0.34
6	70	20	80	1.0	0.36
7	90	5	120	1.0	0.40
8	90	10	80	1.5	0.42
9	90	20	100	0.5	0.46
k_1[b]	0.32	0.35	0.36	0.36	
k_2	0.35	0.37	0.38	0.37	
k_3	0.43	0.38	0.35	0.36	
Range	0.11	0.03	0.03	0.01	
Optimized scheme	A_3	B_3	C_2	D_2	

[a] T represents the total content of four alkaloids in *Corydalis decumbens* (Thunb.) Pers. sample.
[b] k represents the average values of the same level of the same factor.

3.3. Optimization of extraction conditions

An orthogonal experiment was employed in order to optimize the extraction conditions. Four factors were involved: (A) extraction solvent; (B) extraction time; (C) extraction temperature and (D) sample amount. The experimental factors, corresponding levels and orthogonal designs $L_9(3^4)$ are presented in Table 2. The total content of four major alkaloids in *C. decumbens* (Thunb.) Pers. was used as a criterion for the selection of the optimal extraction conditions.

According to the statistical analysis shown in Table 2, the largest range of the three factors was 0.11 of factor A, and the smallest was 0.01 of factor D. This indicated that factor A was the primary factor in the extraction conditions of alkaloids in *C. decumbens* (Thunb.) Pers. samples. The third level of factor A had the largest average value (k_3 = 0.43) compared to the other two levels. This suggested that the third level had the best condition for factor A. By analogy, the third level of factor B, the second level of factor C and the second level of factor D were the best conditions. Thus, we selected the most suitable conditions considering additional criteria, and the optimum extraction conditions were as follows. 1.0 g of finely powdered sample was extracted with 90% ethanol at 100 °C to avoid possible solvent concentration, save energy and minimize interferential co-extraction. An extraction time of 20 min was selected to achieve maximum throughput.

Table 3
Effect of extraction times on extraction efficiency of 4 alkaloids in *Corydalis decumbens* (Thunb.) Pers. (%).

Extraction time	Tetrahydropalmatine	Palmatine hydrochloride	Protopine	Biscuculline
1	97.35 ± 1.56	98.12 ± 1.21	97.05 ± 1.07	97.24 ± 1.65
2	1.25 ± 0.07	1.32 ± 0.08	1.19 ± 0.05	1.78 ± 0.07
3	0.45 ± 0.02	0.58 ± 0.03	0.51 ± 0.02	0.64 ± 0.02

Finally, the effect of extraction times was investigated by running three consecutive extractions on the same sample. As shown in Table 3, two extraction cycles were sufficient to completely extract the four target components from *C. decumbens* (Thunb.) Pers. To evaluate the repeatability of the extraction procedure, a series of six replicates were performed in the same and different days. The results obtained for all target compounds were within 5.3% RSD.

3.4. Comparison of PLE, UAE, MAE and soxhlet extraction

The extraction efficiency of PLE for the four alkaloids was compared with those obtained by UAE, MAE and soxhlet extraction. The relative yields of the four alkaloids extracted from *C. decumbens* (Thunb.) Pers. samples were compared. Yields of alkaloids from *C. decumbens* (Thunb.) Pers. samples by use of PLE were much higher than those achieved by use of MAE, UAE and Soxhlet extraction (Table 4), and PLE has the advantage of shorter extraction time than the other three, which is the main advantage of PLE over UAE, MAE and soxhlet extraction. So PLE was used in present work.

3.5. Method validation

3.5.1. Linearity and detection limit

Results from the calibration study, LODs and LOQs by HPLC–MS/MS for four alkaloids of tetrahydropalmatine, palmatine hydrochloride, protopine and bicuculline are summarized in Table 5. The correlation coefficients were between 0.9993 and 0.9998. The instrumental limits of detection (LODs) were calculated as 3 times the standard deviation; they were 0.02–0.2 $\mu g\,L^{-1}$ for tetrahydropalmatine, palmatine hydrochloride, protopine and biscuculline, respectively. The limits of quantifications (LOQs) were calculated as 10 times the standard deviation; they were 0.07–0.66 $\mu g\,L^{-1}$ for tetrahydropalmatine, palmatine hydrochloride, protopine and biscuculline, respectively for *C. decumbens*

Table 4
Effect of extraction methods on extraction efficiency of 4 alkaloids in *Corydalis decumbens* (Thunb.) Pers. (%).

Extraction method	Tetrahydropalmatine	Palmatine hydrochloride	Protopine	Biscuculline
UAE	86.34 ± 1.65	88.27 ± 1.46	85.24 ± 1.21	90.11 ± 1.61
MAE	89.15 ± 1.32	91.52 ± 1.71	92.38 ± 1.36	92.43 ± 1.45
PLE	97.21 ± 1.35	97.46 ± 1.39	97.68 ± 1.27	97.51 ± 1.32
Soxhlet	88.52 ± 1.23	90.15 ± 1.46	91.29 ± 1.55	90.37 ± 1.57

Table 5
Calibration curve, linear range and detection limits of 4 alkaloids determined by HPLC–MS/MS.

Alkaloids	Calibration curve	Correlation coefficient	Linear range ($\mu g\,L^{-1}$)	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)
Bicuculline	$y = 4.12 \times 10^3 x + 955$	0.9993	1.0–20	0.12	0.40
Protopine	$y = 5.68 \times 10^3 x + 1.57 \times 10^3$	0.9997	1.0–20	0.20	0.66
Tetrahydropalmatine	$y = 2.1 \times 10^4 x + 4.53 \times 10^3$	0.9998	1.0–20	0.02	0.07
Palmatine hydrochloride	$y = 4.37 \times 10^3 x + 476$	0.9996	1.0–20	0.14	0.47

Table 6
Spiked recoveries and RSDs of 4 alkaloids spiked in three levels (%, $n = 3$).

Compound	1 μg L^{-1}		5 μg L^{-1}		10 μg L^{-1}	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
Bicuculline	94.8	3.3	102.6	3.6	98.1	2.4
Protopine	93.6	3.8	103.1	2.9	96.4	2.6
Tetrahydropalmatine	96.5	2.5	103.5	2.7	102.1	2.7
Palmatine hydrochloride	97.4	3.1	98.5	3.1	101.8	2.5

(Thunb.) Pers. samples, which are better than those reported using HPLC and NACE–ESI-MS [13–16].

3.5.2. Precision, reproducibility and stability

The precision of MS/MS peak area measurements was found to be better than 1.63% (RSD, $n = 6$), and the retention time was better than 0.11% for all the target alkaloids. The reproducibility (RSD) of the proposed method based on six replicate injections, were in the range of 2.58–2.72%. The variation of the retention times of all the peaks was less than 0.14% for six replicate injections. The storage stability (RSD) of the measurements for the four alkaloids is 2.18–2.52% ($n = 6$).

3.5.3. Recovery

Recoveries for the four alkaloids were determined by HPLC–MS/MS using standard addition method in which three levels analyses of the spiked samples were run within the same day. The results are summarized in Table 6. The recoveries are within the range of 93.6–103.5%; and the RSD values of all the four alkaloids from three replicate injections are better than 4.0%, demonstrating the good recovery and precision of the method.

3.6. Real samples

The developed HPLC–MS/MS method has been applied to identify and quantify the four alkaloids in different *C. decumbens* (Thunb.) Pers. samples. Each sample was extracted and analyzed in triplicate. Representative MRM chromatogram of the extracts of one *C. decumbens* (Thunb.) Pers. sample is shown in Fig. 2, with the corresponding enhanced product ion (EPI) spectrums of four alkaloids are shown in Fig. 3A–D. The yields of individual alkaloids are summarized in Table 7. Among the four alkaloids, protopine is the most abundant alkaloid in *C. decumbens* (Thunb.) Pers. Our results show that the content of the four major alkaloids in six *C. decumbens* (Thunb.) Pers. samples are noticeably different. Variations might have occurred due to various factors such as geographical source, cultivation, harvest, storage and processing of the herb.

4. Concluding remarks

The pressurised liquid extraction (PLE) provides an efficient and reliable method for the quantitative recovery of four major alkaloids from *C. decumbens* (Thunb.) Pers. A rapid and efficient method for simultaneous identification and quantification of four alkaloids in *C. decumbens* (Thunb.) Pers. by LC–MS/MS was established, which can facilitate the convenient and rapid quality control of the production procedure of *C. decumbens* (Thunb.) Pers. The coupling of LC separation with tandem mass spectrometry provides an attractive tool for the identification of alkaloid compounds in complex natural products. Moreover, the availability of the linear ion trap fragment patterns for each analyte enables a comfortable component confirmation in complex samples.

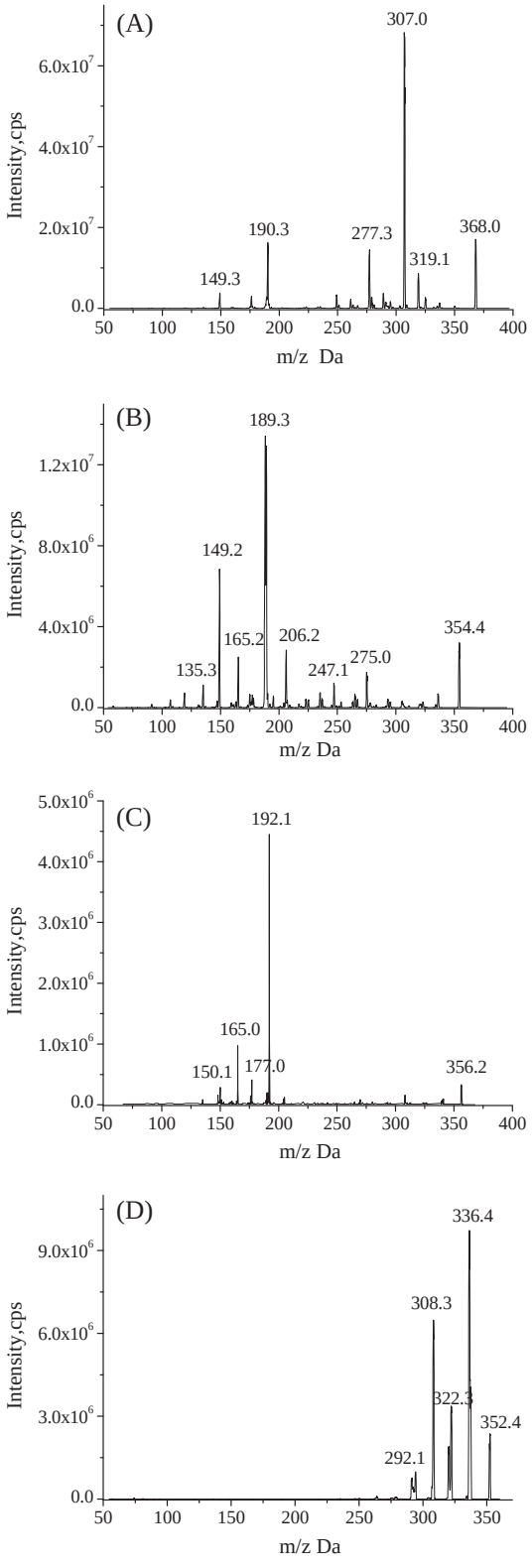

Fig. 3. Corresponding enhanced product ion spectrum of bicuculline (A), protopine (B), tetrahydropalmatine (C), and palmatine hydrochloride (D).

Table 7
Content of 4 alkaloids in different *Corydalis decumbens* (Thunb.) Pers. samples (g kg^{-1}, n = 3).

No.	Tetrahydropalmatine	RSD (%)	Palmatine hydrochloride	RSD (%)	Protopine	RSD (%)	Biscuculline	RSD (%)
1	0.93	3.31	1.02	2.36	1.47	2.93	1.08	3.36
2	0.98	3.56	1.13	2.79	1.63	3.52	1.15	3.08
3	0.85	2.87	0.95	3.54	1.38	2.86	0.91	3.49
4	1.12	2.45	0.85	3.92	1.41	3.41	0.75	2.87
5	1.06	3.24	1.22	4.03	1.05	3.06	1.03	2.62
6	0.92	3.48	1.08	3.68	1.26	3.27	0.88	3.15

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