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Structural analyses of protoberberine alkaloids in medicine herbs by using ESI–FT-ICR-MS and HPLC–ESI–MS^n

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Abstract

The Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI–FT-ICR-MS) using sustained off-resonance irradiation (SORI)/collision-induced dissociation (CID) method at high mass resolution has been first applied to investigate the characteristic fragment ions of four protoberberine alkaloids in medicine herbs. The ESI–FT–ICR SORI-CID experiment results demonstrate that the unambiguous elemental composition of fragment ions can be obtained at high mass resolution, then the logical fragmentation pathways of the protoberberine alkaloids has been proposed. The characteristic fragment ions of CID and MS^n of protoberberine alkaloids have been discussed, which are specific and useful for the identification of some protoberberine alkaloid compounds. Then, the extracts of four kinds of medicine herbs have been analyzed by HPLC–ESI–MS^n. According to these characteristic fragmentation pathways, the retention time (t_R) of HPLC and mass spectra of product ion, the structures of six kinds of protoberberine alkaloids have been identified. And, in the present paper, the selected ion monitoring (SIM) method has been used to separate and identify the alkaloid isomers.

Keywords: Structural analyses; Protoberberine alkaloids; ESI–FT-ICR-MS; HPLC–ESI–MS^n

1. Introduction

Protoberberine alkaloids belong to benzyltetrahydroisoquinolines alkaloids, which widely distribute in the plant kingdom, e.g. in Ranunculaceae plant *Coptis chinensis* Franch., Rutaceae plant *Phellodendron amurense* Rupr., Berberidaceae plant *Berberis poiretii* Schneid and B. *amurensis* Rupr. These four herbs are effectively used in Traditional Chinese Medicine (TCM) for heat clearing, damp drying, and so on [1]. Recently, the most of research results demonstrated that protoberberine alkaloids have wide biological activities [2–5]. In addition, it is very significant to modify the natural alkaloid's structure [1], for example, the natural coralyne has the activity of lowering blood pressure while its derivation compounds can be used for antileukaemia. Thus, it appeared important for us to investigate the constituents of herbs in order to search for new bioactive compounds. Up to now, the identification and investigation of protoberberine alkaloids have been reported by using high-performance liquid chromatography (HPLC) [6,7], high-speed counter current chromatography (HSCCC) [8], and capillary electrophoresis-mass spectrometry (CE–MS) [9,10]. With the development of "soft" ionization technique, electrospray ionization tandem mass spectrometry (ESI–MS^n) has been widely employed to characterize alkaloids owing to its high sensitivity, rapid analysis time and low levels of sample consumption, and meanwhile, the useful structure information for the identification of compounds can be obtained by HPLC–ESI–MS^n [11], which is a powerful analysis tool in the field of phytochemistry [12–14].

In the present paper, the alkaloid standards have been firstly determined by ESI–FT-ICR-MS^n and ESI–MS^n in the positive ion mode, the ions of molecular species M^+ and their MS^n spectra data have been obtained. As a result, the useful

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and characteristic fragment ions have been found. The identification and elucidation of the structure of the protoberberine alkaloids in the four medicine herbs have been done by ESI–MS^n and HPLC–ESI–MS/MS. By comparing the retention time (t_R) and mass spectra of product ions of the alkaloids with authentic standards or literature data, the structures of six kinds of protoberberine alkaloids have been identified. Finally, the quantitation analyses of berberine and palmatine have been done by UV technique. The HPLC–UV–ESI–MS^n method for the identification and quantitative analyses of protoberberine alkaloids in TCM is established, which is important and useful for controlling medicine herbs quality.

2. Experimental

2.1. Apparatus

HPLC system is consisted of a Waters (Milford, MA, USA) 2690 HPLC with a photodiode-array detector set at 277 nm. The chromatographic conditions are as follows: column, Dikma Diamonsil C18, 250 mm × 4.6 mm, 5 μm; eluent, (A) water with 0.0034 mol/l ammonium acetate and 0.2% acetic acid (v/v) and (B) acetonitrile. The linear gradient is 0–60 min 30–88% B; the flow-rate is 0.5ml/min; and the temperature is 23 °C.

The mass spectrometry determination was performed on an LCQ ion trap instrument (Finnigan MAT, San Jose, CA, USA) with an electrospray source in the positive ion mode. The electrospray voltage is set to 5.0 kV. The capillary temperature is 260 °C. HPLC is connected to the mass spectrometer via the UV cell outlet. Selected ion monitoring (SIM) of *m*/*z* 336 and *m*/*z* 338 was employed for further confirmation of alkaloid isomers, respectively.

The high-resolution mass spectrometry was performed using IonSpec Ultima 7.0 T FTICR–MS (IonSpec, USA) with an electrospray source in the positive ion mode. Probe heater is 120 °C. Source heater is 80 °C. Probe HV is set to 3.4 kV. Sample cone voltage is set to 30 V. Extractor cone is set to 5.0 V. Desolvent gas is set to 3.0 V. Cone gas is set to 0.5 V. SORI R_f is set to 2.6 V, and collision gas is N_2 with 100 ms pulse (40 Torr).

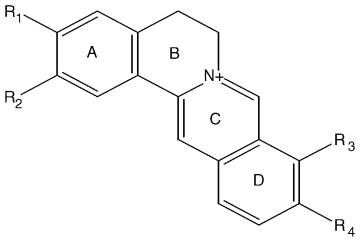
2.2. Standards of alkaloids

Standards of berberine (1), palmatine (2), and jatrorrhizine (3) were purchased from the Chinese Authenticating Institute of Material Medical and Biological Products (Peking, China) and coptisine (4) was kindly provided by Pharmaceutical College of Jilin University. The structures of alkaloids in this study were shown in Fig. 1.

2.3. Sample preparation

2.3.1. Quantitation

The external standard method of calibration was used to the quantitative analyses of the protoberberine alkaloids. Standard solution of berberine was prepared in methanol at the concentrations of 8, 18, 14, 30, and 36 μg/ml, the pamaltine standard solution was prepared in methanol at the concentrations of 5, 10, 15, 20, and 25 μg/ml. The calibra-



	Name	M.W.	R_1	R_2	R_3	R_4
1	berberine	336	-OCH_2O-		OCH_3	OCH_3
2	palmatine	352	OCH_3	OCH_3	OCH_3	OCH_3
3	jatrorrhizine	338	OCH_3	OH	OCH_3	OCH_3
4	coptisine	320	-OCH_2O-		-OCH_2O-	
5	columbamine	338	OH	OCH_3	OCH_3	OCH_3
6	epiberberine	336	OCH_3	OCH_3	-OCH_2O-	

Fig. 1. Structures of alkaloids (1–6) studied.

Table 1
The ESI–FT-ICR-MS^2 data of four alkaloids

Name	Proposed formula	Observed mass (Da)	Calculated mass (Da)	DBE	Error (ppm)	Proposed neutral loss
Berberine	$C_{19}H_{15}O_4N^{\bullet+}$	321.10010	321.09956	13	1.7	$^{\bullet}CH_3$
	$C_{19}H_{14}O_4N^+$	320.09288	320.09228	13.5	−2.3	CH_4
	$C_{18}H_{14}O_3N^+$	292.09779	292.09737	12.5	1.4	C_2H_4O
Palmatine	$C_{20}H_{19}O_4N^{+\bullet}$	337.12972	337.13086	12	−3.4	$^{\bullet}CH_3$
	$C_{20}H_{18}O_4N^+$	336.12338	336.12358	12.5	−0.6	CH_4
	$C_{19}H_{18}O_3N^+$	308.12844	308.12867	11.5	−0.7	C_2H_4O
Jatrorrhizine	$C_{19}H_{17}O_4N^{\bullet+}$	323.11417	323.11521	12	−3.2	$^{\bullet}CH_3$
	$C_{19}H_{16}O_4N^+$	322.10663	322.10793	12.5	−4.0	CH_4
	$C_{18}H_{16}O_3N^+$	294.11191	294.11302	11.5	−3.7	C_2H_4O
Coptisine	$C_{18}H_{14}O_3N^+$	292.09716	292.09737	12.5	0.7	CO

Table 2
The ESI–FT-ICR-MS^3 data of the ion $[M-CH_3]^{+\bullet}$ of three alkaloids

Name	Proposed formula	Observed mass (Da)	Calculated mass (Da)	DBE	Error (ppm)	Proposed neutral loss
Berberine	$C_{19}H_{14}O_4N^+$	320.09190	320.09228	13.5	−1.2	$^{\bullet}H$
	$C_{18}H_{14}O_3N^+$	292.09699	292.09737	12.5	−1.3	$^{\bullet}HCO$
Palmatine	$C_{20}H_{18}O_4N^+$	336.12119	336.12358	12.5	−7.1	$^{\bullet}H$
	$C_{19}H_{18}O_3N^+$	308.12686	308.12867	11.5	−5.9	$^{\bullet}HCO$
Jatrorrhizine	$C_{19}H_{16}O_4N^+$	322.10618	322.10793	12.5	−5.4	$^{\bullet}H$
	$C_{18}H_{16}O_3N^+$	294.11158	294.11302	11.5	−4.9	$^{\bullet}HCO$

tion curves were drafted by the UV peak area versus the compound's concentration. The recovery was determined by standard additions to the plant powder, then the powder was extracted as the method as followed.

2.3.2. Extraction and separation

Coptis chinensis Franch., *Phellodendron amurense* Rupr., *Berberis poiretii* Schneid and B. *amurensis* Rupr. purchased in the Changchun Drug Store were powdered and extracted with 75% of ethanol, respectively. The extractions were repeated three times. After combining them together, the extract solution was passed through a 0.45 μm filter and 3 μl of the filtrate was injected into the HPLC–MS system directly for each herb, respectively.

3. Results and discussion

3.1. The investigation of alkaloid standards by ESI–FT-ICR-MS^n and ESI–MS^n

In the present paper, Berberine (1), palmatine (2), jatrorrhizine (3), and coptisine (4) were first analyzed by using ESI–FT-ICR-MS^n to obtain characteristic ions, and the

Table 3
The CID and MS^n data of four protoberberine alkaloid standards

Compound M^+	CID data					ESI–MS^n data	
	$[M-CH_4]^+$	$[M-2CH_3]^+$	$[M-C_2H_4O]^+$	$[M-2CH_3{-}CO]^+$	$[M-CO]^+$	MS^2	MS^3
Berberine 336	320	306	292	278		321 (−CH_3) → 320 (−H), 292 (−CHO) 306 (−$2CH_3$) 292 (−C_2H_4O) → 277 (−CH_3)	
Palmatine 352	336	322	308	294		337 (−CH_3) → 336 (−H), 308 (−CHO) 322 (−$2CH_3$) 308 (−C_2H_4O) 293 (−CH_3), 278 (−CO)	
Coptisine 320					292	292 (−CO) 274 (−CH_2O_2)	
Jatrorrhizine 338	322	308	294	280		323 (−CH_3) → 322 (−H), 308 (−CH_3), 307 (−CH_4), 294 (−CHO), 279 (−CHO−CH_3) 308 (−$2CH_3$) 294 (−C_2H_4O) → 279 (−CH_3)	

plausible fragmentation pathways were proposed. In the full scan mass spectra, these four alkaloids containing one nitrogen atom exhibited the molecular ions M^+ as even number, so the molecular weight can be easily determined (see Table 1). To further characterize the structures of these compounds, tandem mass spectrometry (MS^n) using SORI-CID technique was applied to analyze these four alkaloids. The experimental results demonstrate that this kind of isoquinoline alkaloids exhibits some common fragment ions in the ESI–FT-ICR-MS^n spectrum, which is shown in Table 1 and Table 2. The structures have been proposed for each of the fragment ions according to their precise mass/charge ratios, and then logical fragmentation pathway of protoberberine alkaloids was proposed. The primary product ions' proposed formula, observed and calculated mass, double bond equivalents (DBE), proposed neutral loss and error of four alkaloids in ESI–FT-ICR-MS^n are summarized in Table 1 and Table 2.The fragmentation pathway of the alkaloid berberine (1) will be discussed as an example to show the fragmentation rule of this kind of alkaloids. In ESI–FT-ICR-MS^2, berberine (1) with two-methoxyl groups substitution at C9 and C10 displayed ions at *m*/*z* 321.10010, *m*/*z* 320.09288,

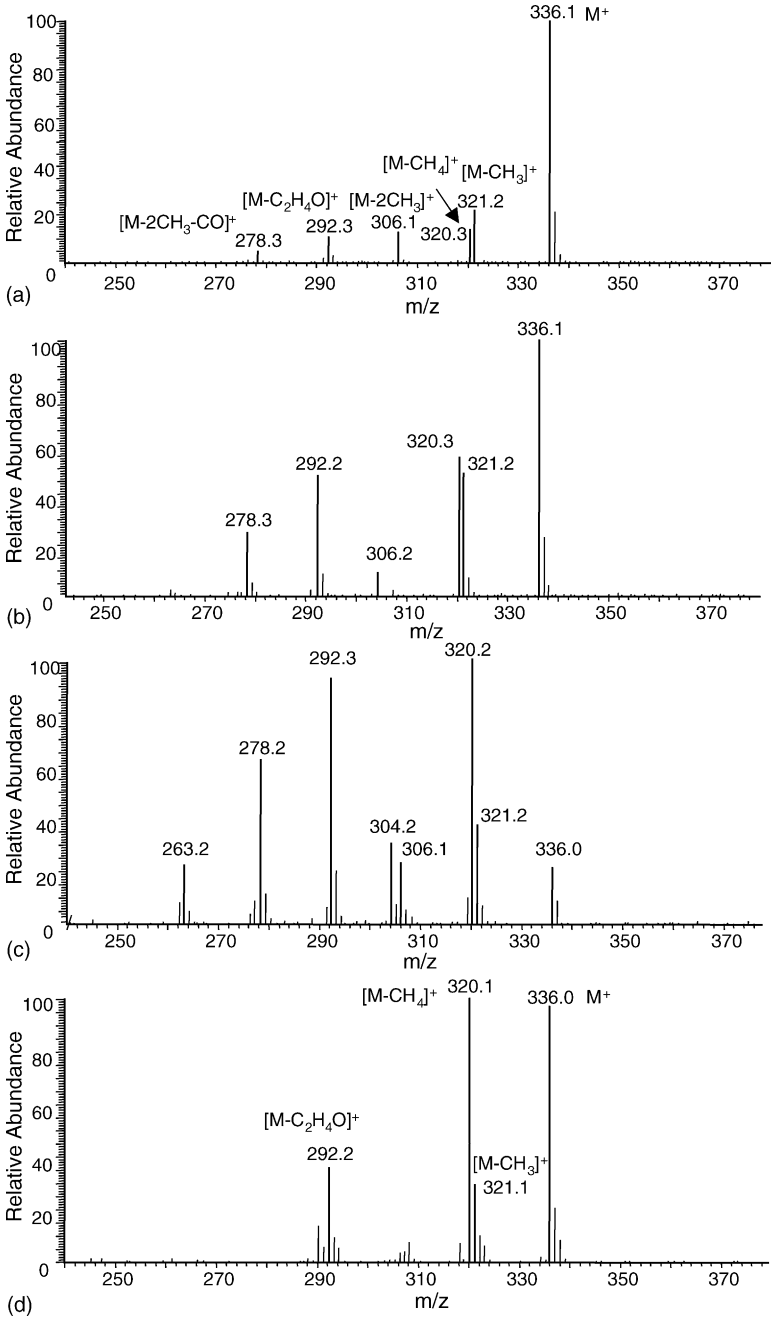


Fig. 2. The CID spectra of berberine in different collision energy (a) 15%, (b) 25%, (c) 50%, and (d) the CID spectrum of epiberberine in 25% collision energy.

and *m*/*z* 292.09779, respectively. The ion at *m*/*z* 321.10010 was determined as the neutral loss of the radical group $^{\bullet}CH_3$ from C9 or C10, the ion at *m*/*z* 320.09288 can be produced by loss of one CH_4, and then the carbonyl group can be formed in C9 or C10 position of D ring via the molecule rearrangement. The ion at *m*/*z* 292.09779 was produced by the loss of C_2H_4O. In ESI–FT-ICR-MS^3, the ion at *m*/*z* 321.10010 lost one $^{\bullet}H$ group to produce the ion at *m*/*z* 320.09228, which has the conjugate structure, and lost one $COH^{\bullet}$ group to form the ion at *m*/*z* 292.09699, which further demonstrates that the ion at *m*/*z* 292.09779 in ESI–FT-ICR-MS^2 was produced by the loss of the neutral molecule C_2H_4O. In ESI–FT-ICR-MS^n experiment, all of alkaloids produced ions $[M-CH_3]^{\bullet+}$, $[M-CH_4]^+$ and $[M-C_2H_4O]^+$ except for coptisine (4), and the ion $[M-CH_3]^{\bullet+}$ exhibited as base peak. The neutral loss of 44 Da was determined as loss of C_2H_4O, with error ranging from −3.7 to 1.4 ppm using FTICR–MS at high mass resolution (Table 1). It is worth noting that compounds (1), (2) and (3) with two-methoxyl groups substitution at C9 and C10 display the common ions of $[M-CH_3]^{\bullet+}$, $[M-CH_4]^+$ and $[M-C_2H_4O]^+$, whereas coptisine with methylenedioxyl group substitution only exhibits ion $[M-CO]^+$. So, it can be concluded that the ions $[M-CH_3]^{\bullet+}$, $[M-CH_4]^+$ and $[M-C_2H_4O]^+$ are the char-

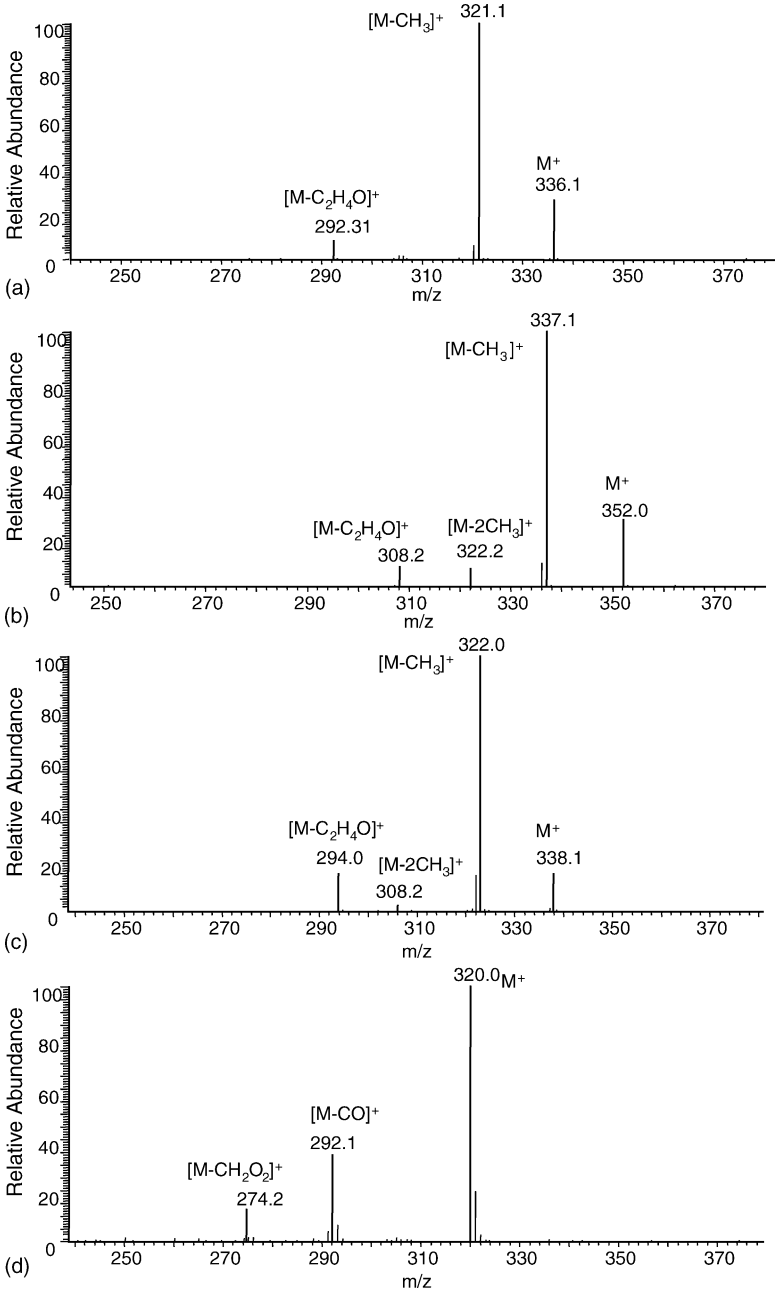


Fig. 3. MS^2 spectra of (a) berberine, (b) pamaltine, (c) jatrorrhizine, and (d) coptisine.

acteristic ions of methoxyl group substituted protoberberine alkaloids.

On the other hand, protoberberine alkaloids have been analyzed by ESI–MS^n. The product ions in ESI–MS^2 are similar to those in the ESI–FT-ICR-MS SORI-CID spectrum. The fragment ions observed by CID in low collision energy (15%) exhibited commonness, all compounds produced $[M - CH_3]^{\bullet+}$ ions except for coptisine (4). However, as the collision energy was increased, the ion signal intensity of the ion $[M - CH_3]^{\bullet+}$ increased. While the higher collision energy (50%) is used, all of alkaloids except for coptisine produced $[M - CH_4]^+$ ions as the base peak. So, it can be seen that hydrogen atom transferring to fragment ions mainly depends on the collision energy. The CID mass spectrum of berberine, as an example, is shown in Fig. 2.

The ESI–MS^n experiments were also employed for the four alkaloids. All of alkaloids (1), (2) and (3) displayed $[M - CH_3]^{\bullet+}$ ion as the base peak, no matter what collision energy was used in the ESI–MS^2 experiment (see Fig. 3, Fig. 4). For coptisine, the ion $[M - CH_3]^{\bullet+}$ was also not observed, while the ion $[M - CO]^+$ at *m/z* 292 exhibited as the base peak. The ions $[M - 2CH_3]^+$ and $[M - C_2H_4O]^+$ were also observed for alkaloids (1), (2), and (3), which are consistent with those ions produced in CID. The fragmentation ions of alkaloids in ESI–MS^n and CID are summarized in Table 3.

3.2. The analyses of the extracts of four medicine herbs

The alkaloids in four medicine herbs were identified in the present paper, and the most characteristic fragment ions were obtained by CID and ESI–MS^n. Peaks 1, 2, 3, 4, and 5 were all observed in the four medicine herbs, while peak 6 was only found in *Coptis chinensis* Franch. As an example, HPLC–UV chromatogram and HPLC–ESI–MS extracted total ion current chromatogram of *Coptis chinensis* Franch. are shown in Fig. 5. Peaks 1 and 2 eluted at 25.4 and 21.4 min in Fig. 5a are easily identified as berberine and palmatine by comparing the retention time, M^+ ions and the characteristic fragment ions with those data of corresponding authentic standards, which all exhibited the characteristic ions $[M - CH_4]^+$ and $[M - C_2H_4O]^+$ in CID experiment. Peak 3 eluted at 17.5 min

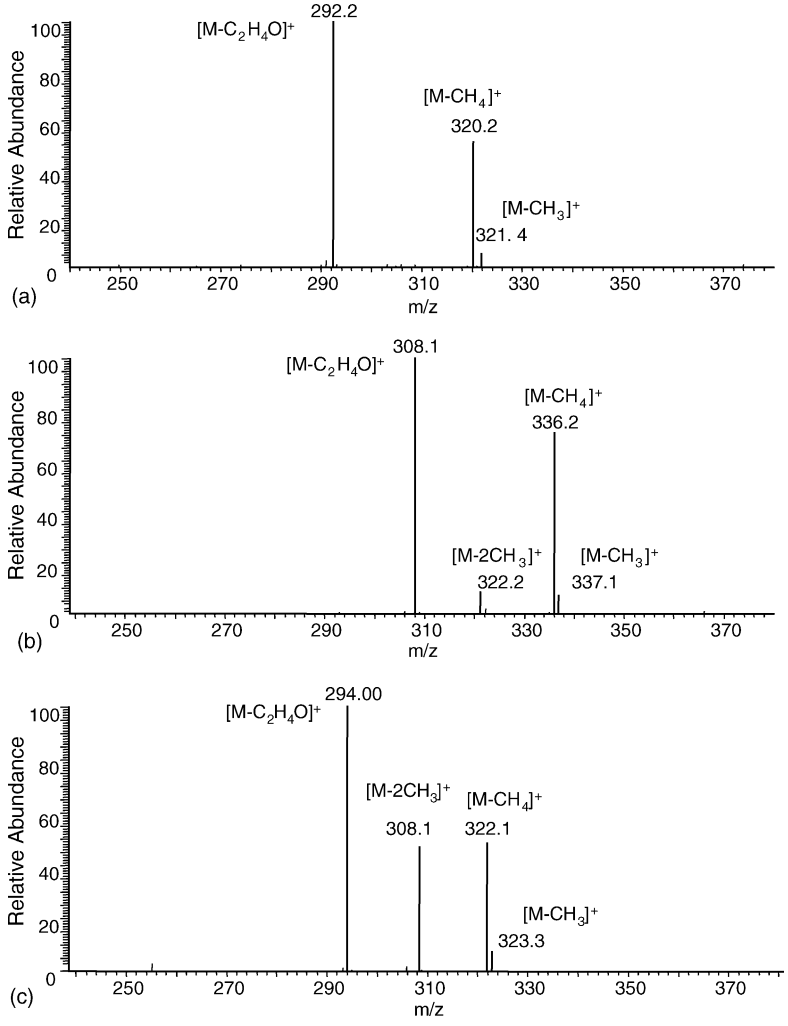


Fig. 4. MS^3 spectra of $[M - CH_3]^+$ of (a) berberine, (b) pamaltine, and (c) jatrorrhizine.

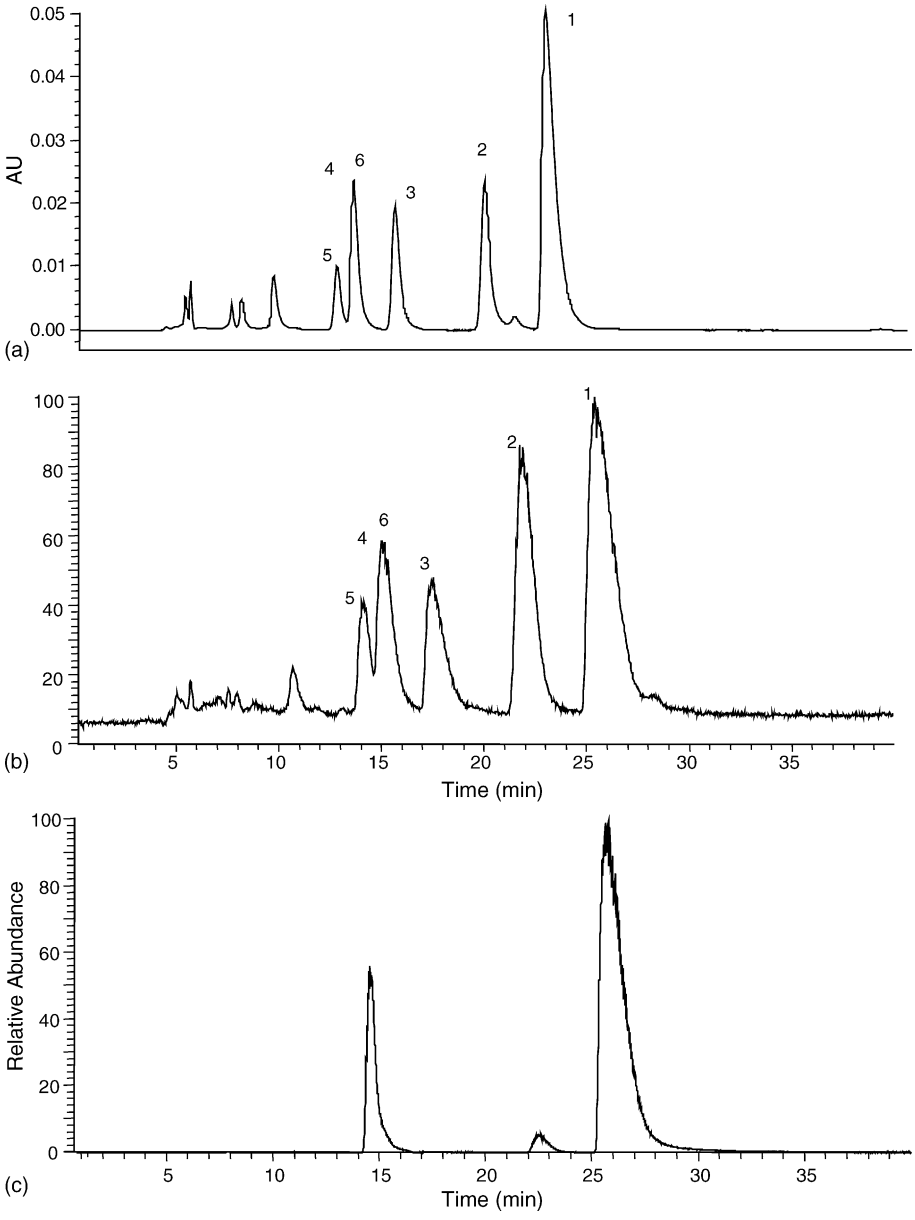


Fig. 5. (a) HPLC–DAD chromatogram of the extract of *Coptis chinensis* Franch., (b) HPLC–MS total ion chromatogram of the extract of *Coptis chinensis* Franch., and (c) SIM ion current chromatogram of ion at *m*/*z* 336 of *Coptis chinensis* Franch.

in Fig. 5a, exhibited the M^+ ion at *m*/*z* 320 as the base peak in the HPLC–ESI–MS, and the specific ion $[M - CO]^+$ was produced in CID and ESI–MS^n experiment. In addition, its retention time is also consistent with the standard coptisine. According to the above information, the compound of peak 3 was confirmed as coptisine. The peak observed at 15.0 min in Fig. 5a, corresponds to the ions at *m*/*z* 338 and at *m*/*z* 336 in ESI–MS, which is named as peaks 4 and 6, respectively. For the ion at *m*/*z* 338 named as peak 4, the retention time and the fragment ions obtained by CID and ESI–MS^n are consistent with the information of jatrorrhizine standard (Table 4). And meanwhile, for the ion at *m*/*z* 336 named as peak 6, the product ions in ESI–MS^n are the same as those of berberine, while the CID data of peak 6, shown in Fig. 2d, are different from those of berberine. The experimental results demonstrate that the ion at *m*/*z* 278 was not found in the CID product ion spectrum of peak 6, which is formed by the loss of $2CH_3 + CO$ from berberine ion in ESI–MS^2, On the contrary, the characteristic ion $[M - CO]^+$ at *m*/*z* 308 was obtained, which is the characteristic ion of the isoquinoline alkaloids with C9 and C10 methylenedioxy substitution. So, according to the literatures [15,16], the compound of peak 6 was identified as epiberberine. Because of the methoxyl group substitution at C2 and C3 position in the A ring, epiberberine is not easy to lose two CH_3 groups to form conjugated structure with the B ring, because the B ring is not in conjugation, as a result, in low energy collision dissociation experiment, the ion at *m*/*z* 278 was not observed. The compound of peak 5 eluted at

14.0 min in Fig. 5a, also yields the ion at *m*/*z* 338 and analogous fragment ions of jatrorrhizine in CID and ESI–MS^n. According to the literature [15], the compound of peak 5 can be identified as columbamine.

In order to further identify the two kinds of isomer ions at *m*/*z* 336 and at *m*/*z* 338, the selected ion monitoring method was used to get the SIM ion current chromatogram of ions at *m*/*z* 336 and at *m*/*z* 338. The ion at *m*/*z* 336 in *Coptis chinensis* Franch. was first selected to be monitored and the experiment results are shown in the Fig. 5c. From Fig. 5c, it can be seen that two peaks were observed at 15.0 and 25.4 min, respectively, which are consistent with the retention time of peak 6 corresponding to epiberberine and peak 1 corresponding to berberine. When the ion at *m*/*z* 338 for *Coptis Chinensis* Franch. was selected to be monitored, two peaks at 14.0 and 15.0 min were observed, respectively, (Fig. 6a), which are consistent with peak 4 corresponding to jatrorrhizine and peak 5 corresponding to columbamine in Fig. 5a. So, it is demonstrated that the SIM experiment could provide further proofs for the determination of alkaloids in medicine herbs.

As mentioned above, the protoberberine alkaloids, i.e., berberine, palmatine, coptisine, jatrorrhizine, and columbamine can be identified and elucidated by compared with their retention times, fragment ions data of CID and ESI–MS^n of standards and literature, which are displayed in Table 4. The SIM ion current chromatograms of the ion at *m*/*z* 338 for

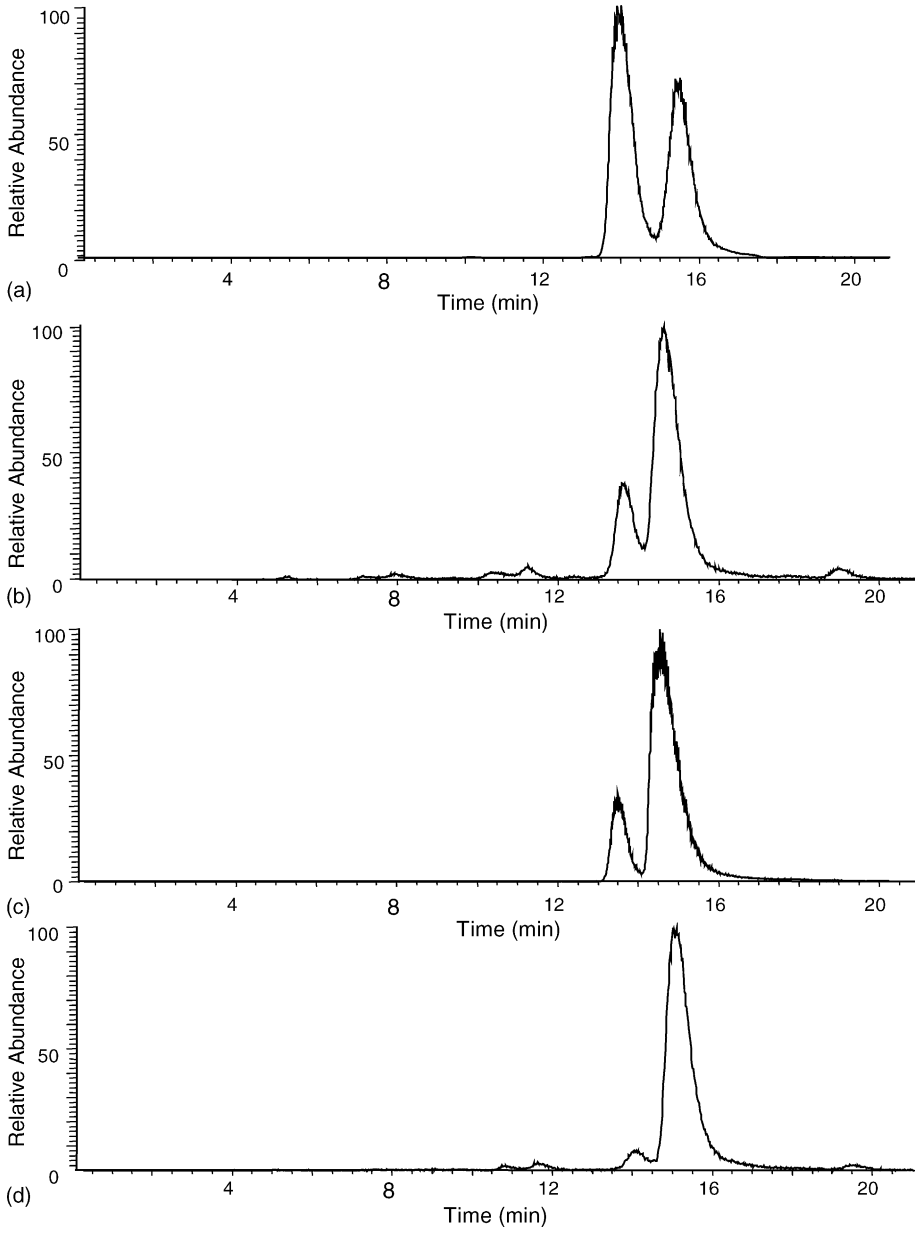


Fig. 6. SIM ion current chromatograms of the ion at *m*/*z* 338 for (a) *Coptis chinensis* Franch., (b) B. *amurensis* Rupr., (c) *Berberis poiretii* Schneid, and (d) *Phellodendron amurense* Rupr.

Table 4
The HPLC–ESI–MS^n data of identified alkaloids

Peak no.	t_R	M	CID data	ESI–MS^n data		Compound deterrminend	Herbs
				MS^2	MS^3		
1	25.4	336	320, 306, 304, 292, 278	321 → 320, 292 306 292 → 277		Berberine	a–d
2	21.4	352	336, 322, 308, 294	337 → 336, 322, 308 322 308 → 292, 278		Palmatine	a–d
3	17.5	320	292	292 274		Coptisine	a–d
4	15.0	338	322, 308, 294, 280	323 → 322, 308, 307, 294, 279 308 294 → 279		Jatrorrhizine	a–d
5	14.0	338	322, 308, 294, 280	323 → 322, 308, 307, 294, 279 308 294 → 279		Columbamine	a–d
6	15.0	336	320, 308, 292	321 → 320, 318, 292 306 292 → 278		Epiberberine	a

a: Coptis chinensis Franch., b: Phellodendron amurense Rupr. c: Berberis poiretii Schneid, and d: B. amurensis Rupr.

Table 5
The quantitative content of berberine and pamaltine in the extracts of medicine herbs

Herb	Berberine (mg/g)	Pamaltine (mg/g)	Average recovery (%)
Coptis chinensis Franch.	91.54	27.61	87
Phellodendron amurense Rupr.	10.22	5.480	86
Berberis poiretii Schneid	0.9327	0.3407	87
B. *amurensis* Rupr.	0.4338	0.3738	88

B. *amurensis* Rupr., *Berberis poiretii* Schneid and *Phellodendron amurense* Rupr. are shown in Fig. 6, and the difference among the four medicine herbs can be easily found.

3.3. The quantitative analyses of berberine and palmatine in the extracts

The regression equations, linearities, detection limits of berberine, and palmaltine in the extracts are studied. The regression equations for berberine and palmaltine are $Y = 40537X - 33609$ and $Y = 38209X + 11031$, respectively. The quantitative analyses were performed by means of the external standard methods. The results of the quantitative analyses are shown in Table 5.

4. Conclusion

The logical fragmentation pathways of protoberberine alkaloids have been proposed by ESI–FT-ICR-MS, which is very useful for the rapid identification of protoberberine alkaloids. The information of molecular weight and the characteristic fragment ions have been obtained by HPLC-ESI–MS^n. And the identification of protoberberine alkaloids in crude extracts of medicine herbs has been done by compared with the corresponding data of standards or literature. The experimental results demonstrate that the ESI–MS^n technique is a rapidly determination method of the non-isomeric alkaloids, and the CID and SIM method are very helpful for differentiation of isomers. In one word, ESI–MS^n, CID, and HPLC-MS are powerful analytical tools for rapid screening of alkaloids in crude plant extracts without time consuming and tedious separation work.

Acknowledgments

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