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Separation and Identification of the Constituents in Fangchi Radix of Different Origins

WEN-YING HUANG, CHENG-HUNG SU AND SHUENN-JYI SHEU *

Department of Chemistry, National Taiwan Normal University, 88, Sec. 4, Tingchow Rd., Taipei City, Taiwan.

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ABSTRACT

Fangchi Radix is derived from Aristolochiaceae or Menispermaceae plants and contains a series of biologically active alkaloids. Fifteen alkaloids in Fangchi crude extracts, including acutumidine(1), magnoflorine(2), stepharine(3), sinomenine(4), acutumine(5), cyclanoline(6), fangchinoline(7), berbamine(8), tetrandrine(9), isotetrandrine(10), trilobine(11), aristolochic acid Π(12), aristolochic acid I(13), aristololactam(14) and isotrilobine(15), were separated completely by high-performance liquid chromatography (HPLC) within 60 min. UV detection at 254 nm with a linear gradient elution consisting of 20 mM CH_3COONH_4 (pH 4.81) and H_2O-CH_3CN was found to be the most efficient eluent for the LC and LC-MS separations. The structures of the compounds corresponding to the peaks in the LC chromatogram were identified by LC-MS. A total of 32 commercial samples of Fangchi Radix from *Sinomenium acutumn*, *Stephania tetrandra*, *Aristolochia fangchi* and *Cocculus trilobus* were collected from herbal markets in Taiwan, Japan and China. The contents (peak-area ratios) and structures of the fifteen constituents in these samples were readily determined by the proposed methods and the results could be used to estimate the origin and quality of an herbal drug.

Key words: Fangchi Radix, fangchi alkaloids, HPLC, LC-MS

INTRODUCTION

Fangchi Radix, one of the most commonly used Chinese herbal drugs, is derived from the Aristolochiaceae (*Aristolochia fangchi*, *A. heterophylla*) and Menispermaceae plants (*Sinomenium acutumn*, *Stephania tetrandra*, *Cocculus trilobus*, *trilobus*)[1]. Those herbs contain a series of alkaloids, including acutumidine(1), magnoflorine(2), stepharine(3), sinomenine(4), acutumine(5), cyclanoline(6), fangchinoline(7), berbamine(8), tetrandrine(9), isotetrandrine(10), trilobine(11), aristolochic acid Π(12), aristolochic acid I(13), aristololactam(14), and isotrilobine[2-9](15), as shown in Figure 1, and are known to own anti-hypertensive[10], histamine-release inhibitory[11], anti-inflammatory, anti-nociceptive, antipyretic[12-13], anti-rheumatic[14] and pluripotent suppressive[15-16] effects. Several methods, including high-performance liquid chromatography (HPLC), liquid chromatography-mass (LC-MS) and capillary electrophoresis (CE), have been employed for the analysis of these alkaloids[17-21]. However, none is practicable in terms of resolving all the constituents of the extract in a single run.

We describe herein, a simple and rapid HPLC and LC-MS method for the simultaneous determination and identification of these fifteen compounds in 32 samples from four different Fangchi species.

* Author for correspondence. Tel: +886-2-29350749 ext. 405; Fax: +886-2-29309073; E-mail: beca69042014@yahoo.com.tw.

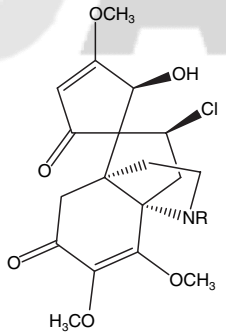
MATERIALS AND METHODS

I. *Reagents and Materials*

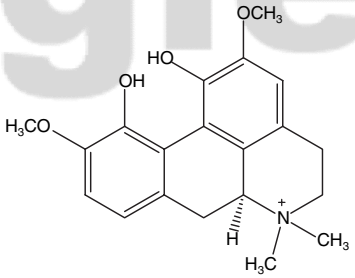
Sinomenine, tetrandrine, aristolochic acid I, aristolochic acid Π were purchased from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan), propyl 4-hydroxybenzoate, from Aldrich (Milwaukee, WIS, USA), acetic acid, acetonitrile, methanol from Merck (Darmstadt, Germany) and ammonium acetate from Nacalai Tesque (Kyoto, Japan). Other marker substances were isolated from Fangchi Radix[1,3,6-9,15,19,22-24]. Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions. Samples of Fangchi Radix were provided by the Brion Research Institute of Taiwan and were identified by their external appearance and pharmacognostic histological anatomy. Thirty-two herb samples, collected from different herbal shops throughout Taiwan, Japan, and China, were examed the same way as above. They were found to be derived from four species, namely *Sinomenium acutumn* (S1-S5), *Stephania tetrandra* (T1-T8), *Aristolochia fangchi* (A1-A13) and *Cocculus trilobus* (C1-C6).

II. *Preparation of Fangchi Radix Extracts*

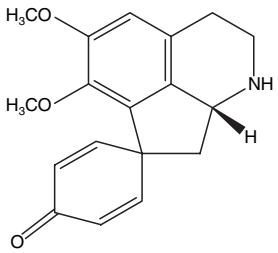
A 1.25-g sample of pulverized Fangchi Radix was extracted by refluxing in 70% methanol (15 mL) for 15 min, followed by centrifugation of the suspension at 1500 *g* (Universal, Hettich Zentrifugen) for 5 min. The



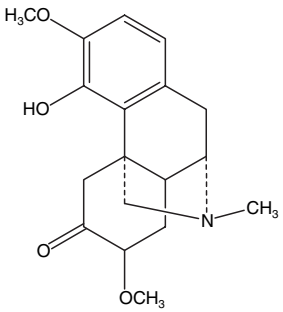
acutumidine(1): R = H
acutumine(5): R = CH_3



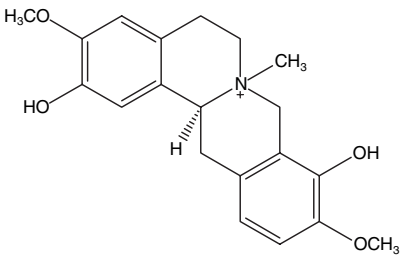
magnoflorine(2)



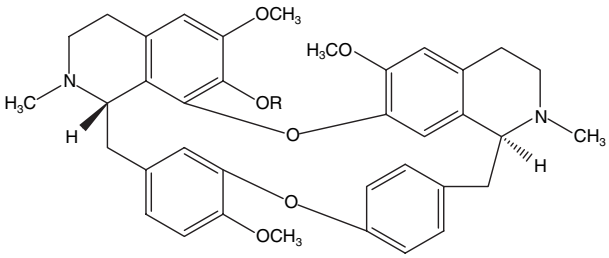
stepharine(3)



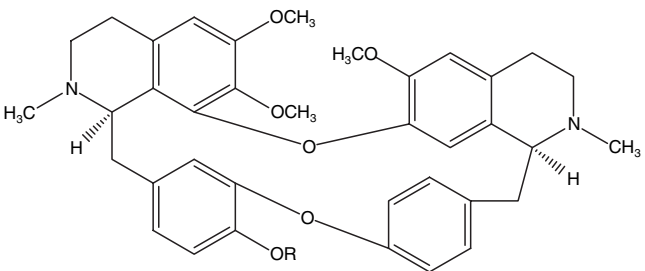
sinomenine(4)



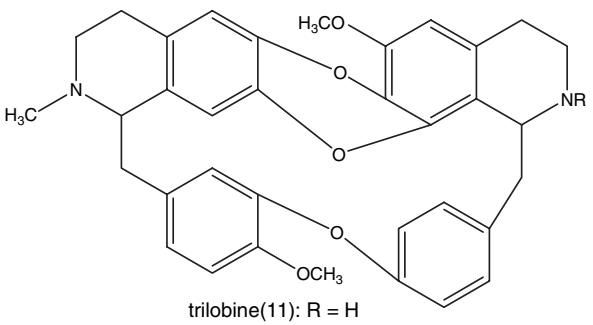
cyclanoline(6)



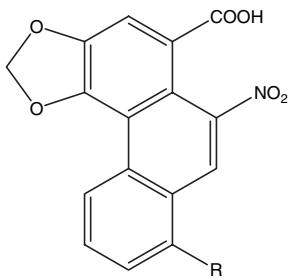
fangchinoline(7): R = H
tetrandrine(9): R = CH_3



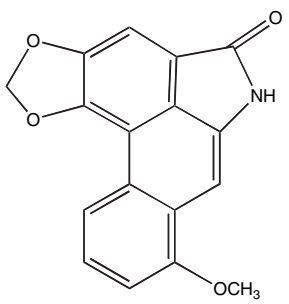
berbamine(8): R = H
isotetrandrine(10): R = CH_3



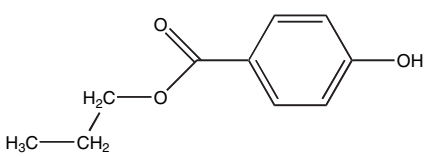
trilobine(11): R = H
isotrilobine(15): R = CH_3



aristolochic acid II (12): R = H
aristolochic acid I (13): R = OCH_3



aristololactam(14)



propyl 4-hydroxybenzoate(IS)

Figure 1. Structures of the fifteen constituents.

extraction was repeated three times. The extracts were combined and filtered through a 0.45-μm filter. After the addition of 1 mL of an internal standard (51.9 mg of propyl 4-hydroxybenzoate in 100 mL of 70% methanol), the extract was diluted to 50 mL with 70% methanol. This solution was passed through a 0.45-μm filter and the filtrate was injected into the HPLC and LC-MS system. Recovery was determined by comparing the amount of marker added with that of the marker found.

III. *Apparatus and Conditions*

(I) *LC-UV System*

HPLC was performed using a Jasco 890 instrument equipped with a Jasco 890 system controller and a Jasco 975 photodiode array detector (254 nm). The substances were separated on a reverse-phase column (Cosmosil $5C_{18}$-MS, 5μm, 25 cm × 4.6 mm I.D.) (Nacalai Tesque, Kyoto, Japan) which was eluted at a flow rate of 1.0 mL/min with a linear solvent gradient of A-B [A = 20mM CH_3COONH_4-CH_3COOH (500:0.25 v/v, pH 4.81; B = H_2O-CH_3CN (20:80 v/v)] using the following protocol: 0 min, 10%B; 10 min, 20%B; 15 min, 25%B; 20 min, 30%B; 25min, 37%B; 30 min, 40%B; 35min, 55%B; 40min, 60%B; 45min, 80%B%; 60min, 10%B.

(II) *LC-MS System*

The compounds were identified using a Thermo Finnigan AQA advantage system (Thermo Electron corporation, San Jose, CA, USA). The AQA included a SPECTRA SERIES P100 system with a SPECTRA SERIES UV-6000LP detector, and was equipped with an electrospray ionization (ESI) interface. The chromatographic conditions were the same as that used for HPLC. After the HPLC UV-detector, eluent was passed through a splitter and entered the AQA ion source at a rate of about 150 μL/min. The ESI source was operated at a sheath gas flow of 6 psi, an electrospray needle of 3 kV, and a heated-capillary temperature of 350°C. The energy level for the CID (collision induced dissociation) fragmentation was maintained at 50%.

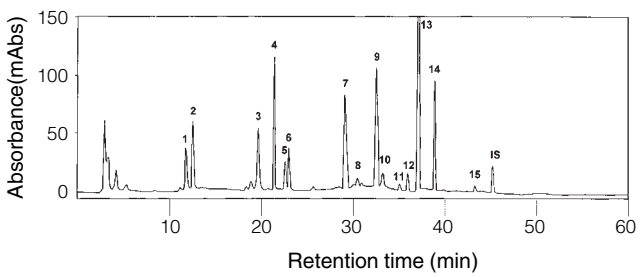


Figure 2. HPLC chromatograms of 15 marker substances in Fangchi Radix.

Table 1. Resolution values for the adjacent peak-pairs in proposed HPLC methods

peak-pair	Rs
(1) / (2)	1.78
(3) / (4)	4.63
(5) / (6)	1.25
(7) / (8)	2.74
(9) / (10)	1.66
(11) / (12)	2.58
(13) / (14)	4.20
(14) / (15)	6.29

RESULTS AND DISCUSSION

I. *HPLC Analysis*

All fifteen constituents and the internal standard were successfully resolved and identified in a single run by the HPLC and LC-MS methods under suitable conditions. Separation was achieved by optimizing the pH of the buffer, the acetate concentration, the analytical column, and the organic modifier. In order to optimize resolution (Rs), four reversed-phase columns, Cosmosil 5C18-MS, 5C18, 5C18-AR and 5C18-AR II were initially examined. Cosmosil 5C18-MS was found to provide the best separation, which gave the highest theoretical plate numbers for all peaks and the best resolution for the pair of compounds acutumidine(1) and magnoflorine(2) (Rs = 5.13). Using 5C18-MS as the analytical column, a series of buffer solutions with different acetate concentrations and pH values were examined. Analyses were performed using 0, 10, 20, 30, 40 and 50 mM solutions of aqueous ammonium acetate and the results showed that 20 mM was optimal for the separation of all the fifteen compounds, especially for the pair of peaks corresponding to isotetrandrine(10) and trilobine(11) in terms of resolution (Rs = 3.54). With the addition of 0.05% acetic acid (v/v, pH 4.81), a well resolved chromatogram for all the compounds was obtained not only from the standpoint of peak sharpness but also the smoothness of the baseline. As a result, a solution consisting of 20 mM ammonium acetate and acetic acid was used as the mobile phase A.

Mobile phase B composed of water with variable concentration of acetonitrile and methanol. Higher concentration of acetonitrile caused the constituents to elute earlier, whereas higher methanol ratios caused the less polar constituents unable to elute within the analysis time. Regarding the resolution of tetrandrine(9)/isotetrandrine(10), various ratios of acetonitrile/methanol/water (80/0/20, 60/20/20, 45/45/10, 30/60/10 and 0/80/20) were examined. The results indicated that the resolution of this peak-pair increased with increasing ratio of acetonitrile. At a ratio of 80/0/20, the resolution of (9)/(10) was optimal (Rs = 1.66), and the solution, therefore, was chosen to be mobile phase B (Table 1). Using mobile phase (A) = 20

mM CH_3COONH_4 (pH 4.81) with 0.05% CH_3COOH and mobile phase (B) = CH_3CN/H_2O = 80/20 (v/v) in a gradient elution, chromatograms for the four species of Fangchi samples were obtained and shown in Figure 3. There was no significant interference with the peaks for samples from any of the species.

A validation test of the method showed that this proposed LC method resulted in good precision and accuracy for all peaks, and good linearity for 4 authentic compounds, (4), (9), (12) and (13). The reproducibility (relative standard deviation) of the peak-area ratio was 0.74-1.46% (intraday, n = 6) and 0.88-1.65% (interday, n = 6) and of the retention time was 0.07-0.83% (intraday, n = 6) and 0.20-1.25% (interday, n = 6). The recoveries of the components ranged 98.1 to 101.2%. Detailed data are shown in Table 2. The detection limits (S/N = 3) for the authentic components were 0.08 μg/mL for (4), sinomenine, 0.75 μg/mL for (9), tetrandrine, 0.46 μg/mL for (12), aristolochic acid II, 0.46 μg/mL for (13), aristolochic acid I. The concentration range of each calibration curves was 1.75-280 μg/mL for (4), 1.50-240 μg/mL for (9), 0.91-145 μg/mL for (12), 0.91-145 μg/mL for (13).

II. *LC-MS Analysis*

The fifteen compounds analyzed in this study are mainly alkaloids. Since the HPLC separation and qualitative MS analyses must be carried out simultaneously for all compounds, it is necessary to select an ion source suitable for the analyses. Hence, we compared the complete TIC charts of the APCI positive, negative ion modes (APCI ±) and ESI positive, negative ion modes (ESI ±) and tried

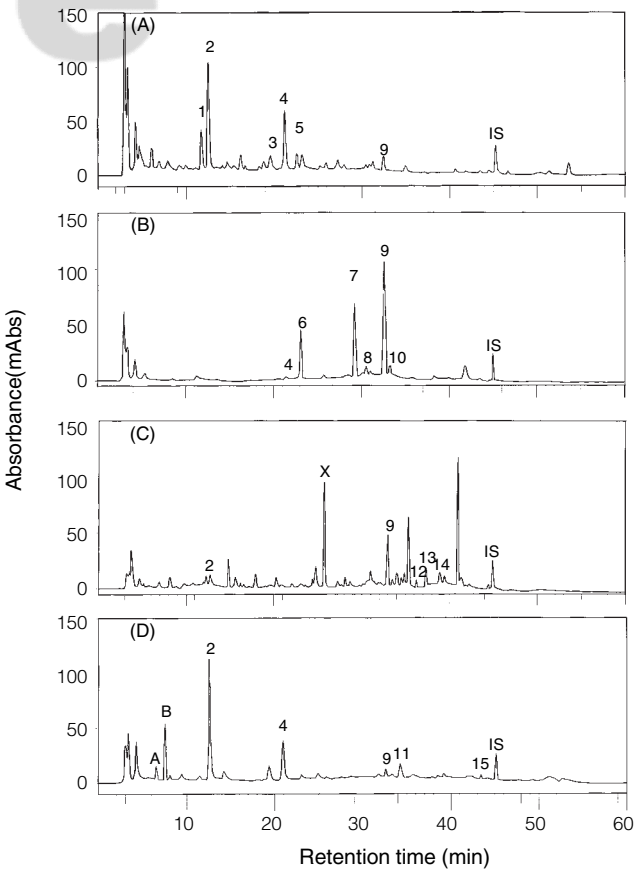


Figure 3. HPLC chromatograms of extracts of Fangchi samples. (A) *Sinomenium acutumn* extract; (B) *Stephania tetrandra* extract; (C) *Aristolochia fangchi* extract; (D) *Cocculus trilobus* extract. Peaks A, B and X are unknown compounds.

Table 2. Reproducibility under HPLC analysis conditions

compound	Reproducibility (RSD, %)			
	Intraday(n = 6)		Interday(n = 6)	
	Peak-area ratio	Retention time	Peak-area ratio	Retention time
(1)	1.06	0.69	1.49	1.12
(2)	1.42	0.67	1.65	1.24
(3)	1.09	0.83	1.34	1.12
(4)	1.14	0.57	1.16	1.17
(5)	1.04	0.52	1.15	1.05
(6)	1.18	0.44	1.21	1.09
(7)	1.06	0.82	1.31	1.15
(8)	1.14	0.67	1.16	1.23
(9)	1.46	0.18	1.58	0.72
(10)	1.03	0.12	1.17	0.42
(11)	0.93	0.15	1.05	0.46
(12)	0.98	0.15	1.16	0.34
(13)	0.83	0.27	0.91	0.39
(14)	0.74	0.07	0.88	0.20
(15)	1.21	0.57	1.33	1.25

Journal of Food and Drug Analysis, Vol. 14, No. 4, 2006

to determine one ion source suitable for all of the fifteen compounds. It was found that the TIC from ESI yielded a greater abundance of MS peaks than APCI, and that the ESI positive ion mode yielded a better resolution and lower noise level. Therefore, ESI was used for the ion source, and cations were monitored. The resulting MS spectrum then served as the source of data for the qualitative analysis.

III. *Identification of the Constituents*

Fifteen constituents, including 4 commercially available and 11 isolated compounds, in the Fangchi samples, were well separated by the proposed method and their structures were determined by comparing the LC-MS data with those reported in the literature (Figure 4-7). The major MS peaks of these compounds are as follows: acutumidine(1), gave a base peak at m/e = 384 for $[M+H]^+$ and had, therefore, a molecular weight of 383[3]; magnoflorine(2), $[M]^+$=342, molecular weight 342[6]; stepharine(3), $[M+H]^+$=298, molecular weight 297[7]; sinomenine(4), $[M+H]^+$=330, molecular weight 329[22], [compared with authentic standard]; acutumine(5), $[M+H]^+$=398, molecular weight 397[23]; cyclanoline(6), $[M]^+$=342, molecular weight 342[8]; fangchinoline(7), $[M+H]^+$=608, molecular weight 607[7]; berbamine(8), $[M+H]^+$=608, molecular weight 607[24]; tetrandrine(9), $[M+H]^+$=623, molecular weight 622[22], [with authentic standard]; isotetrandrine(10), $[M+H]^+$=622, molecular weight 621[24]; trilobine(11), $[M+H]^+$=563, molecular weight 562[9]; aristolochic acid Π(12), $[M+H-H_2O]^+$=294, molecular weight 311[19], [with authentic standard]; aristolochic acid I(13), $[M+H-H_2O]^+$=324, molecular weight 341[5], [with authentic standard]; aristololactam(14), $[M+H]^+$=294, molecular weight 293[1]; isotrilobine(15), $[M+H]^+$=577, molecular weight 576[9]. UV spectra acquired from a photodiode array detector and the elution order postulated according to the molecular polarity were also used to confirm the assignments.

IV. *Origins of the Samples and Contents of the Constituents*

Thirty-two Fangchi samples were analyzed by the proposed method and some representative chromatograms were shown in Figure 3. Figure 3 revealed that *Sinomenium acutumn* contained no(6), (7), (8), (10), (11), (12), (13), (14), (15), *Stephania tetrandra* no(1), (2), (3), (5), (11), (12), (13), (14), (15), *Aristolochia fangchi* no(1), (3), (4), (5), (6), (7), (8), (10), (11), (15), and *Cocculus trilobus* no(1), (3), (5), (6), (7), (8), (10), (12), (13), (14) (Table 3). The contents of all the constituents, the peak-area of each constituent divided by peak-area of the internal standard, in four different species were calculated and shown in Table 4. It is also shown in Table 4 that substantial differences in the constituents among the samples and the species exist. Compounds acurumidine(1), stepharine(3) and acutumine(5) were found to be present only in *Sinomenium acutumn* with the contents (peak-area ratios) of (1), 0.809-1.408, (3), 0.649-1.019 and (5), 0.407-0.865. Compounds cyclanoline(6), fangchinoline(7), berbamine(8) and isotetrandrine(10) were only present in *Stephania tetrandra* samples with contents of (6)2.202-4.765, (7)2.292-4.102, (8)0.372-1.073 and (10)0.116-0.199. *Aristolochia fangchi* contained special

Table 3. Presence of the constituents in four species of Fangchi samples

constituents	Fangchi Radix sample			
	Sinomenium acutumn	*Stephania tetrandra*	*Aristolochia fangchi*	*Cocculus trilobus*
(1)	◎			
(2)	◎		◎	◎
(3)	◎			
(4)	◎	◎		◎
(5)	◎			
(6)		◎		
(7)		◎		
(8)		◎		
(9)	◎	◎	◎	◎
(10)		◎		
(11)				◎
(12)			◎	
(13)			◎	
(14)			◎	
(15)				◎

◎: The strain contains this constituent

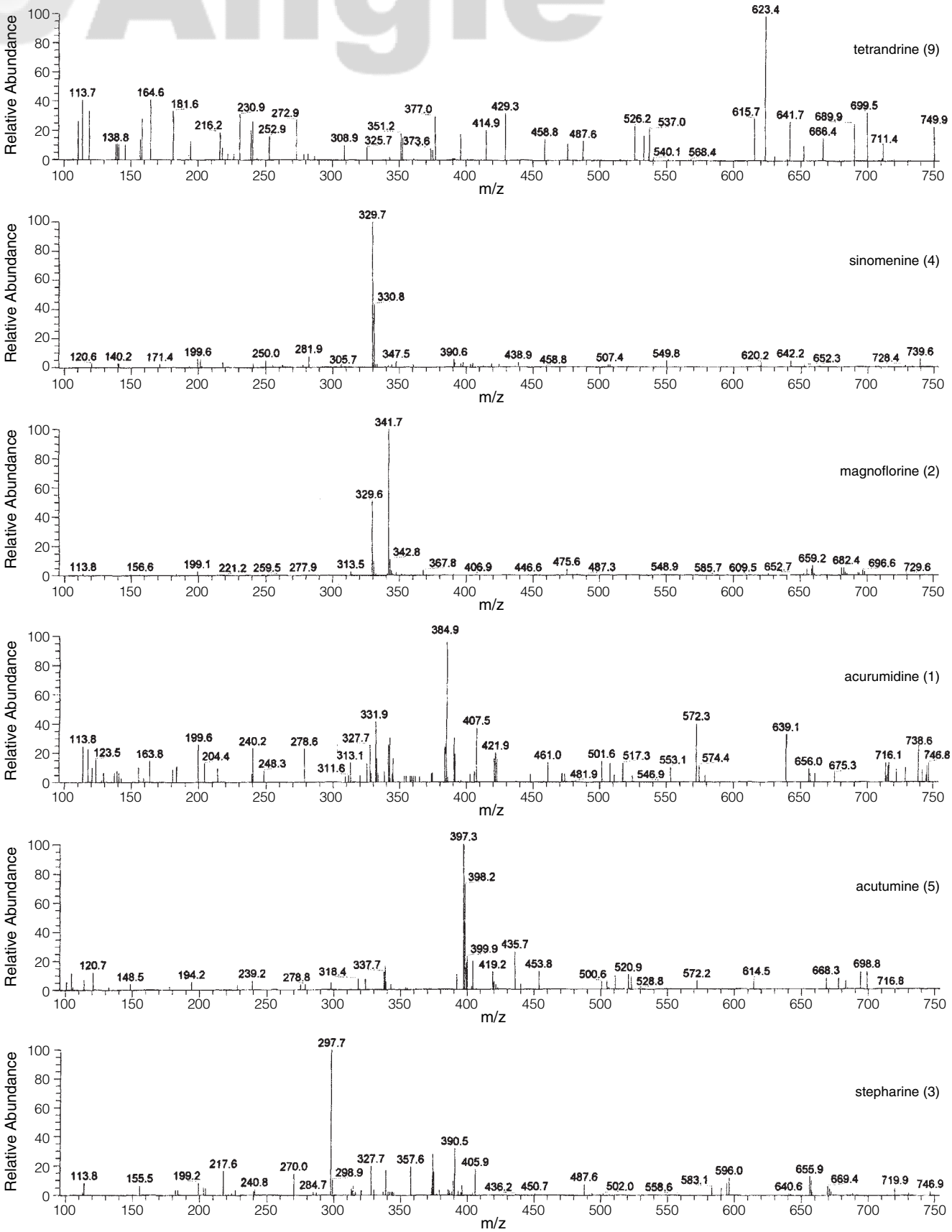


Figure 4. LC-MS fragments for the constituents of *Sinomenium acutumn* extract.

Journal of Food and Drug Analysis, Vol. 14, No. 4, 2006

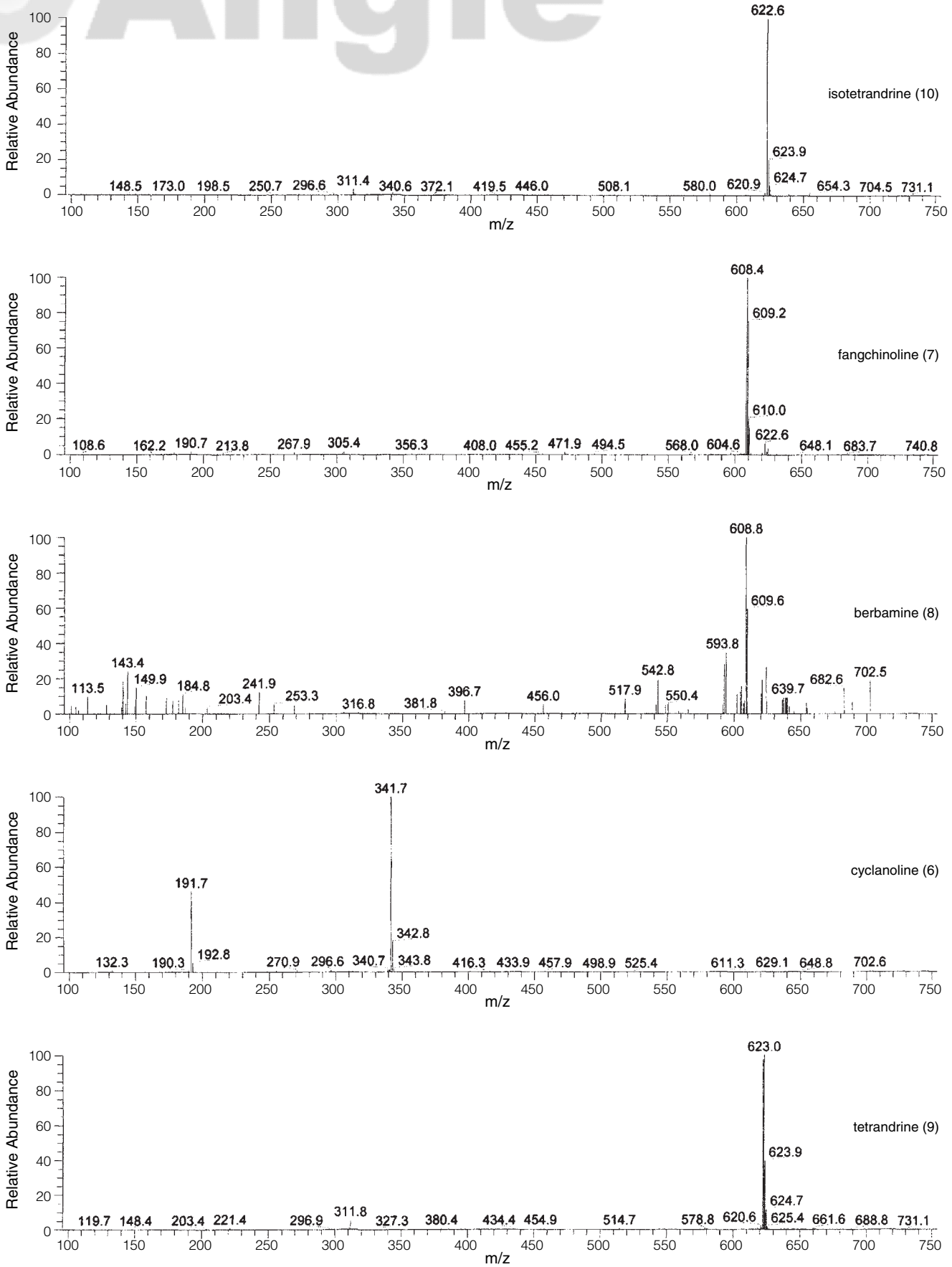


Figure 5. LC-MS fragments for the constituents of *Stephania tetrandra* extract.

constituents, aristolochic acid Π(12), 0.021-0.057, aristolochic acid I(13), 0.275-0.676, aristololactam(14), 0.276-0.595; and only *Cocculus trilobus* contained trilobine(11), 0.539-0.711, isotrilobine(15), 0.060-0.098. These results could be used to differentiate samples derived from different species.

It has been shown recently that the presence of aristolochic acids in Fangchi preparations is the major factors concerning its nephrotoxic and carcinogentic effects[25]. According to the data listed above, only samples from *Aristolochia fangchi* contained aristolochic acid I(13) and aristolochic acid Π(12).

CONCLUSIONS

This work successfully demonstrates that, high-resolution separations of a complicated mixture can be easily

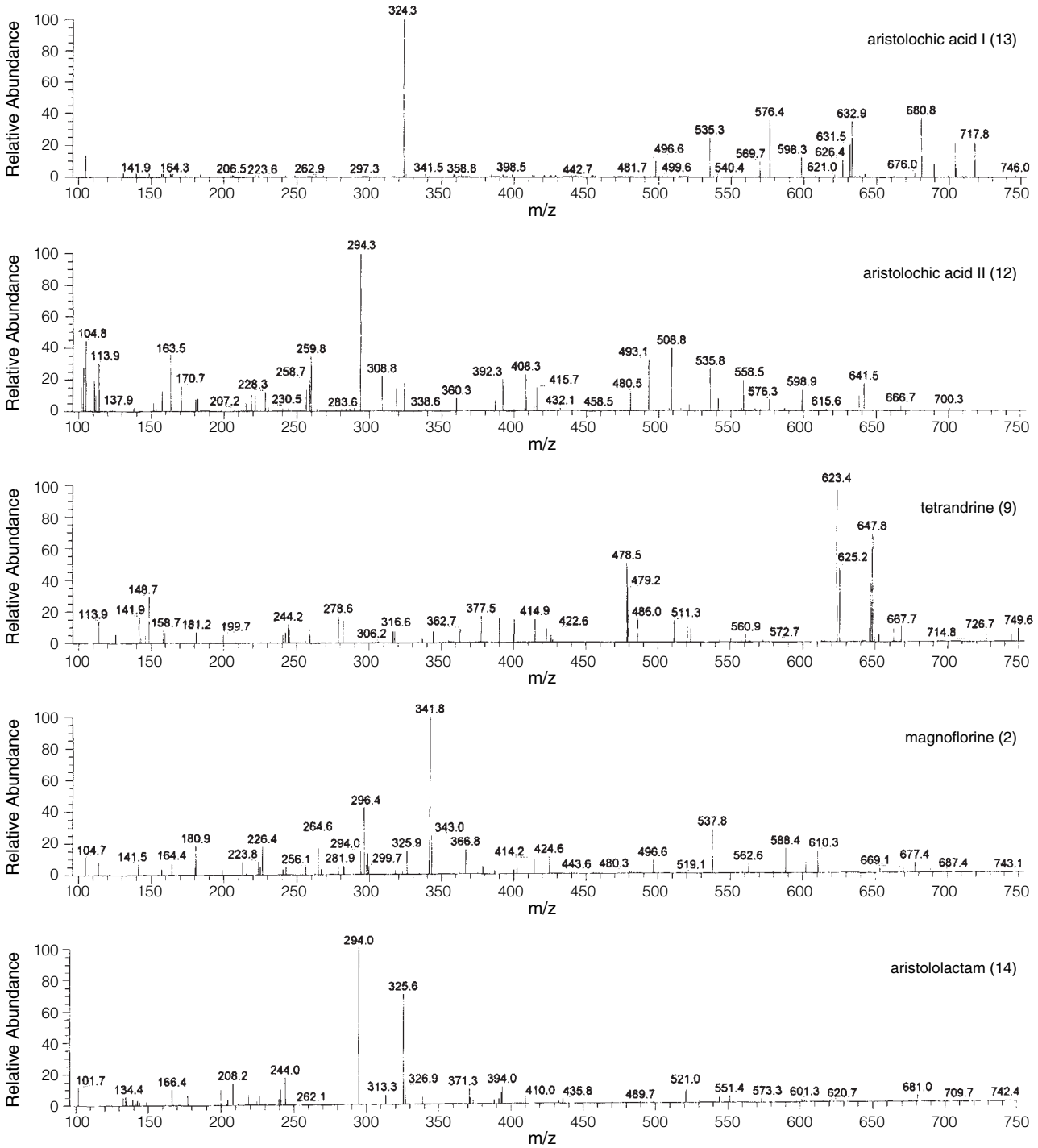


Figure 6. LC-MS fragments for the constituents of *Aristolochia fangchi* extract.

achieved by optimizing parameters such as acetate, acetic acid, methanol, acetonitrile concentration of the eluent system and the column used. By combining LC-UV-ESI-MS systems, this method can be used, not only to recognize authentic standards but also to identify peaks of interest. The species that make up the commercial samples

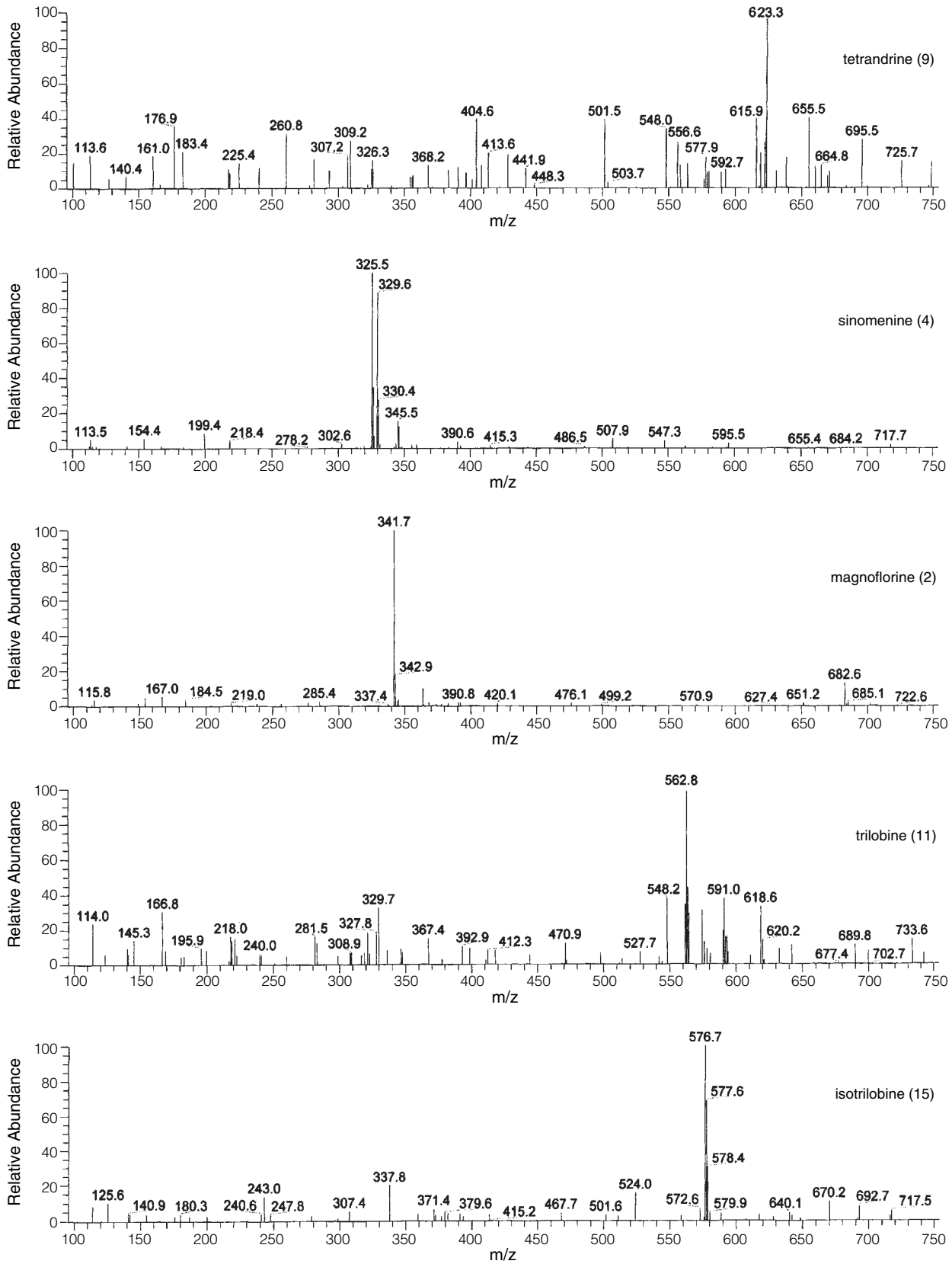


Figure 7. LC-MS fragments for the constituents of *Cocculus trilobus* extract.

Table 4. Peak area ratios of the constituents in Fangchi samples

constituents	Fangchi Radix sample (Ave. ± SD)			
	Sinomenium acutumn	*Stephania tetrandra*	*Aristolochia fangchi*	*Cocculus trilobus*
A / (IS)				0.468 ± 0.092
B / (IS)				1.193 ± 0.098
(1) / (IS)	1.123 ± 0.228			
(2) / (IS)	4.498 ± 0.331		0.140 ± 0.023	8.462 ± 1.064
(3) / (IS)	0.781 ± 0.140			
(4) / (IS)	1.576 ± 0.177	0.144 ± 0.033		0.455 ± 0.059
(5) / (IS)	0.605 ± 0.224			
(6) / (IS)		3.316 ± 1.098		
X / (IS)			2.363 ± 0.544	
(7) / (IS)		3.537 ± 0.641		
(8) / (IS)		0.671 ± 0.241		
(9) / (IS)	0.619 ± 0.148	8.741 ± 1.941	1.716 ± 0.367	0.151 ± 0.032
(10) / (IS)		0.163 ± 0.043		
(11) / (IS)				0.628 ± 0.075
(12) / (IS)			0.039 ± 0.012	
(13) / (IS)			0.527 ± 0.111	
(14) / (IS)			0.457 ± 0.116	
(15) / (IS)				0.073 ± 0.016

1. Average contents of (4) is 1.484 mg/g and (9) is 0.292 mg/g in *Sinomenium acutumn*; (4) is 0.424 mg/g and (9) is 3.64 mg/g in *Stephania tetrandra*; (9) is 0.745 mg/g, (12) is 0.125 mg/g and (13) is 0.221 mg/g in *Aristolochia fangchi*; (4) is 0.668 mg/g and (9) is 0.099 mg/g in *Cocculus trilobus*.
2. Peaks A, B and X are unknown compounds.

could be identified using analytical data obtained from the proposed methods.

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