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Fingerprint analysis of *Psoralea corylifolia* L. by HPLC and LC–MS

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Abstract

High-performance liquid chromatography (HPLC) was developed for fingerprint analysis of *Psoralea corylifolia*. Liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS^n) technique was first employed to identify the components of the fingerprint. The samples were separated with an Alltima C_{18} column (250 mm × 4.6 mm, 5 μm) by linear gradient elution using water–acetic acid (A; 100:0.1, v/v) and acetonitrile (B; 0 min, 40%; 15 min, 50%; 35 min, 60%; 45 min, 70%; 55 min, 80%; and maintained for 5 min) as mobile phase at a flow rate of 1.0 ml/min and detector wavelength at 245 nm. A standard procedure was developed for HPLC fingerprint analysis. Average chromatogram of 10 batches of *P. corylifolia* L. from Sichuan and Henan Provinces, PR China, which has been considered as the original and genuine herbal medicine for a long time, was first established as the characteristic fingerprint. There are 12 common peaks in this fingerprint. Ten of these common peaks were identified by MS data. This profile was then used to identify and assess the differences among the herb grown in various areas of China. The HPLC fingerprint analysis is specific and may serve for quality identification and comprehensive evaluation of *P. corylifolia*.

Keywords: Fingerprint; *Psoralea corylifolia*; HPLC; LC–MS; MS data; Identification

1. Introduction

Recently, chromatographic fingerprint technique, as a more meaningful formulation for controlling the quality of herbal samples or their products, has been attracting more and more people's attention because the fingerprint technique emphasizes on the systemic characterization of compositions of samples and focus on identifying and assessing the stability of the plants. Fingerprint analysis has been introduced and accepted by WHO as a strategy for the assessment of herbal medicines [1]. And it is also required by the Drug Administration Bureau of China to standardize injections made from traditional Chinese medicines (TCM) and their raw materials [2]. The chromatographic methods involving fingerprint include TLC, high-performance liquid chromatography (HPLC), X-ray, CE, etc. Because of its advantages and popularization, HPLC fingerprint analysis has been regarded as the first choice [3–5].

Psoralea corylifolia (Chinese name Buguzhi), dry fruits of leguminous plant *P. corylifolia* L., is one of the most popular TCM and officially listed in the Chinese Pharmacopoeia. This crude drug has been used for the treatment of enuresis, pollakiuria, painful feeling of cold in the waist or knees and weak kidney. The constituents in *P. corylifolia* include coumarins and flavone components, such as psoralen, isopsoralen, psoralidin, bavachalcone, etc. [6,7] This crude drug is grown and used all over China. The content of each component varies significantly due to difference in geographic origin, climate condition, environment and other factors. Determination of mere one or several components is not adequate representative [8,9]. The aim of this study is to develop a characteristic fingerprint of *P. corylifolia* using HPLC for identifying the raw herb.

Liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS^n) has grown into one of

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the most powerful analytical techniques currently available. Among its advantages, LC–ESI-MS^n has provided a high level of sensitivity and selectivity. It is widely used in the analysis of complex mixtures. By coupling LC to an ion-trap mass spectrometer, structural information can be obtained by collision-induced dissociation (CID). In this study, LC–ESI-MS–MS was first utilized to corroborate the structure of the main constituent in the *P. corylifolia* and help to control the quality of *P. corylifolia*. The method provides useful information for quality control of *P. corylifolia* crude drugs.

2. Experimental

2.1. Materials and reagents

P. corylifolia were collected from Sichuan and Henan Provinces, PR China, all of which had been identified as the Genuine Medicinal Herb by Professor Xuehua Song in China Pharmaceutical University.

Psoralen and isopsoralen were provided by National Institute for the Control of Pharmaceuticals and Biological Products, Beijing, China.

Bavachin, corylin, psoralidin, bavachalcone and bavachinin were isolated from *P. corylifolia* in our laboratories. The purity of the isolated components was shown to be higher than 99% analyzed by HPLC, and their identities were confirmed by IR, ^{1}H and ^{13}C NMR.

Ethanol and acetic acid were both analytical grade, and acetonitrile was chromatographic grade.

2.2. Instrumentation and chromatographic condition

The HPLC system consisted of two delivery pumps (Shimadzu LC-10AD, Japan), a UV detector (Shimadzu, LC-10AVP, Japan), and a model 7725i manual injector valve with a 0.02 ml sample loop.

An Agilent 1100 series LC system equipped with a binary pump was connected to an Agilent G1313A autosampler.

Chromatographic separation was carried out at room temperature using an Alltima C_{18} analytical column (250 mm × 4.6 mm, 5 μm) supplied by Alltech (Deerfield, IL). The mobile phase consisted of water–acetic acid (A; 100:0.1, v/v) and acetonitrile (B); A:B was as follows: 0 min, 60:40; 15 min, 50:50; 35 min, 40:60; 45 min, 30:70; 55 min, 20:80; and maintained for 5 min. The flow rate was 1.0 ml/min. The detector wavelength was set at 245 nm.

The mass spectrometry detector (MSD) was equipped with an ESI source. The ionization mode was positive. The interface and MSD parameters were as follows: nebulizer pressure, 25 psi (N_2); dry gas, N_2 (8 l/min); dry gas temperature, 325 °C; spray capillary voltage, 3500 V; skimmer voltage, 40 V; ion transfer capillary exit, 94 V; scan range, *m*/*z* 100–800; spectra average, 5; ion current control, on; target, 30,000; and dwell time, 300 ms. All data acquired were proceeded by Agilent Chemstation Rev. A. 09.01 software (Agilent, Palo Alto, CA).

2.3. Sample preparation

0.2 g sample of the fine-grinded powder was accurately weighted and extracted with 20 ml of 70% ethanol in ultrasonic bath for 30 min at 30 °C and filtered. Five milliliters of the continual filtrate was diluted to 25 ml volumetric flask by the same solvent as sample solution and filtered through a 0.45-μm filter membrane before analysis.

Twenty microliters of the sample solution was injected to HPLC column and separated under above chromatographic conditions.

3. Results and discussion

3.1. Optimization of HPLC systems

Photodiode array detector (DAD) was applied to select the optimized wavelength of constituents in the fingerprint. In a full-scan experiment, chromatogram at 245 nm shows more components information and better separation than at other wavelength. Next, provided that coumarins and flavones are phenolic acid substances, we have added 0.1% of acetic acid in mobile phase in order to prevent dissociation. Because in constant speed elution, some of these components in the sample have a long retention time, we choose linear gradient elution instead. Satisfactory results are obtained within 60 min for the HPLC separation.

3.2. Standardization of fingerprint

The method validation of fingerprint analysis was performed based on the relative retention time (the ratio of peak retention time of sample constituents to the reference standard) and the relative peak area (the ratio of peak area of sample constituents to the reference standard). Among these active components, psoralen indicates a high and stable content, therefore we choose it as the reference substance. All common peaks' relative retention time and relative peak area are obtained on the basis of this substance. The precision of the proposed method, on the basis of analyzing five replicate samples, were below 1.8% and 1.3% for RSD of relative retention time and relative peak area of all peaks, respectively. The reproducibility test was performed with six sample solutions extracted from one batch of plant. The RSD of the relative retention times and the relative peak areas were both less than 3%. The stability test was performed with sample solutions for 24 h. RSD of both, the relative retention times and the relative peak areas, were less than 3%. The result indicates that this method is adequate, valid and applicable.

To standardize the fingerprint, 10 batches of *P. corylifolia* samples from Sichuan and Henan were analyzed with the

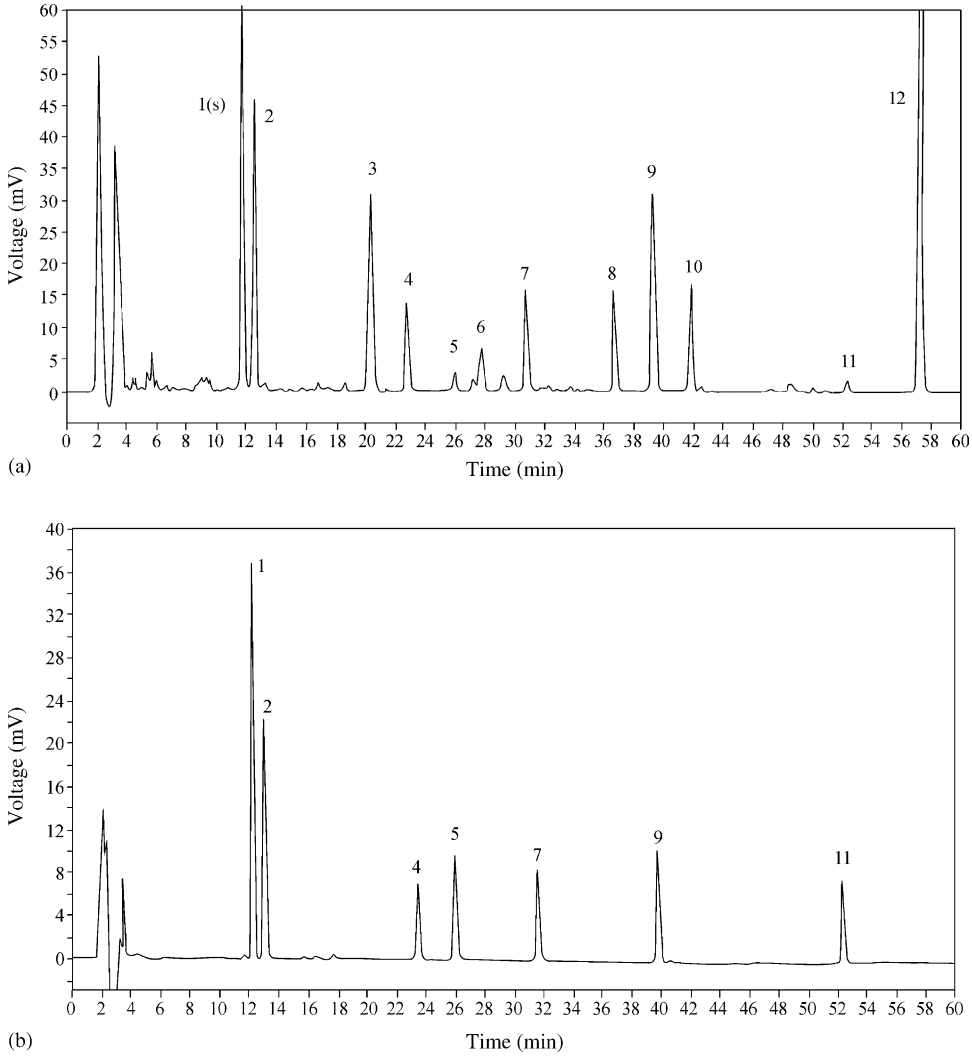


Fig. 1. HPLC chromatograms of average of 10 batches of samples (a) and mixture standard compounds (b): (1) psoralen; (2) isopsoralen; (3) neobavaisoflavone; (4) bavachin; (5) corylin; (6) bavachromene; (7) psoralidin; (8) isobavachalcone; (9) bavachinin; (10) unknown; (11) bavachalcone; (12) unknown.

developed procedure. Peaks that existed in all 10 batches of samples were assigned as "common peaks" for *P. corylifolia*. There are 12 "common peaks" in the fingerprint. The whole chromatogram together with standards could provide useful means of identifying and assessing *P. corylifolia*. Average fingerprint chromatogram of 10 batches of samples is shown in Fig. 1(a) and chromatogram of mixture standard compounds is shown in Fig. 1(b).

Table 1
The mass data and compound names of the 12 peaks

Peak number	Mass data	Identification
1	187 $[M+H]^+$, 395 $[2M+Na]^+$, 159 $[M+H-CO]^+$	Psoralen
2	187 $[M+H]^+$, 395 $[2M+Na]^+$, 159 $[M+H-CO]^+$	Isopsoralen
3	323 $[M+H]^+$, 267 $[M-C_4H_7]^+$, 239 $[M-C_4H_7-CO]^+$, 211 $[M-C_4H_7-2CO]$	Neobavaisoflavone
4	325 $[M+H]^+$, 269 $[M-C_4H_7]^+$	Bavachin
5	321 $[M+H]^+$, 137 seeing below	Corylin
6	323 $[M+H]^+$, 667 $[2M+Na]^+$, 345 $[M+Na]^+$	Bavachromene
7	337 $[M+H]^+$, 281 $[M-C_4H_7]^+$	Psoralidin
8	325 $[M+H]^+$, 269 $[M-C_4H_7]^+$	Isobavachalcone
9	339 $[M+H]^+$, 283 $[M-C_4H_7]^+$	Bavachinin
10	391 $[M+H]^+$, 267, 239	Unknown substance 1
11	339 $[M+H]^+$, 699 $[2M+Na]^+$, 361 $[M+Na]^+$	Bavachalcone
12	586 $[M+H]^+$, 436, 316	Unknown substance 2

3.3. HPLC–MS analysis for components identification

HPLC–ESI-MS^n has been employed to the components analysis in *P. corylifolia* crude drugs. In an ESI-MS experiment, we obtained molecular weight of each peak. Next, from ESI-MS^n data, coupled with ultraviolet absorption, HPLC retention time [10–16] and standard chromatogram, we have deduced 10 possible structures of these 12 common peaks. Because the ionization mode was positive, most of the *m*/*z* data are $[M+H]^+$, $[M+Na]^+$, $[M+Na]^+$ or $[2M+Na]^+$. In this ionization mode, flavones and coumarin combined with isopentenyl would lose this group and indicate a characteristic *m*/*z* data as $[M-55]^+$, from which we can deduce whether the substance have isopentenyl. Moreover, most of flavones and coumarins contains a keto carbonyl group, so their *m*/*z* data are often $[M-C_4H_7]^+$ and $[M+H-CO]^+$, so there would be some $[M-55]^+$, $[M+H-28]^+$ and $[M-55-28]^+$ fragments. The mass data and compound names of the 12 peaks

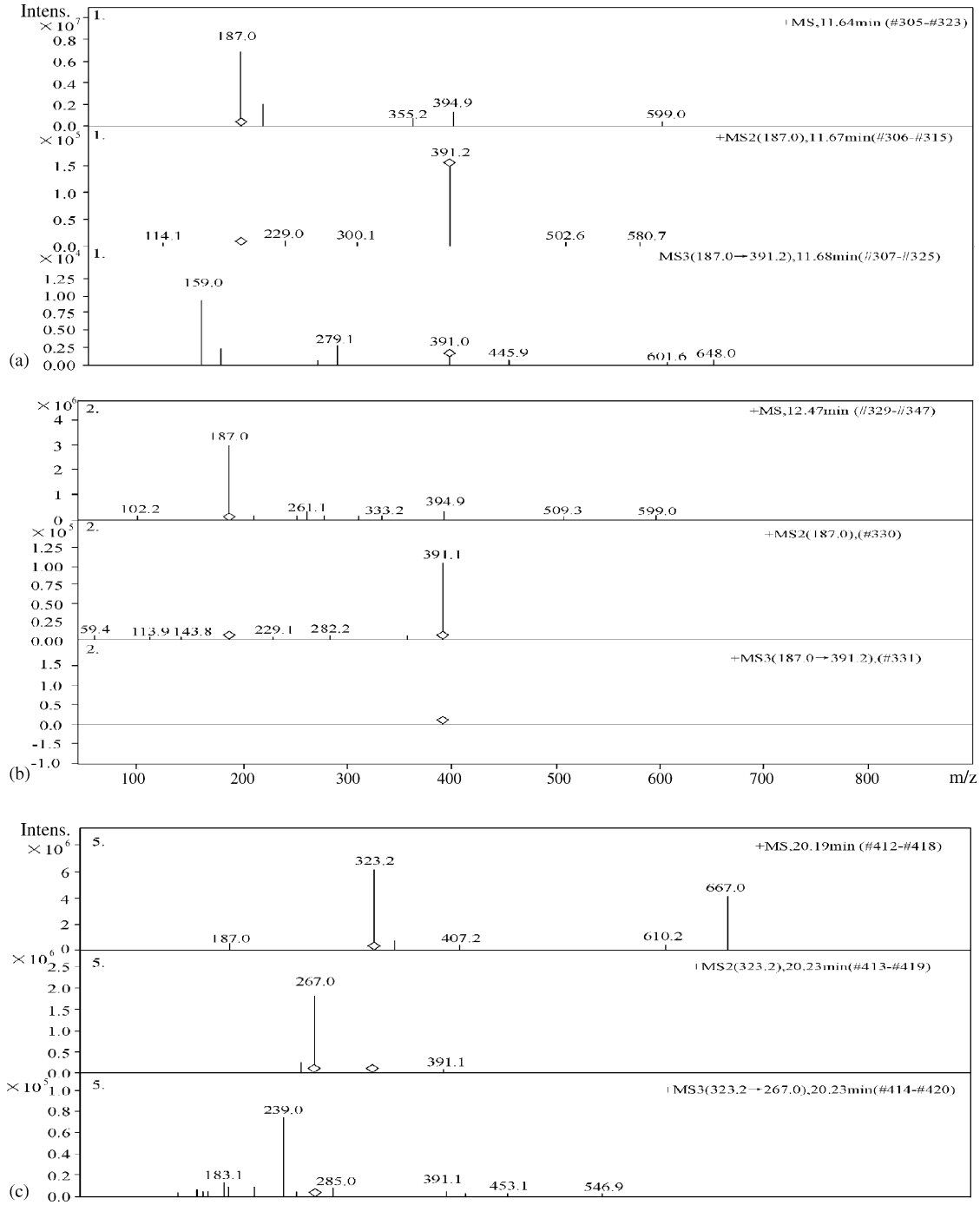


Fig. 2. MS, MS^2 and MS^3 mass spectrum of: (a) psoralen, *m*/*z* 187; (b) isopsoralen, *m*/*z* 187; (c) neobavaisoflavone, *m*/*z* 322; (d) bavachin, *m*/*z* 324; (e) corylin, *m*/*z* 320; (f) bavachromene, *m*/*z* 322; (g) psoralidin, *m*/*z* 336; (h) isobavachalcone, *m*/*z* 324; (i) bavachinin, *m*/*z* 338; (j) bavachalcone, *m*/*z* 338.

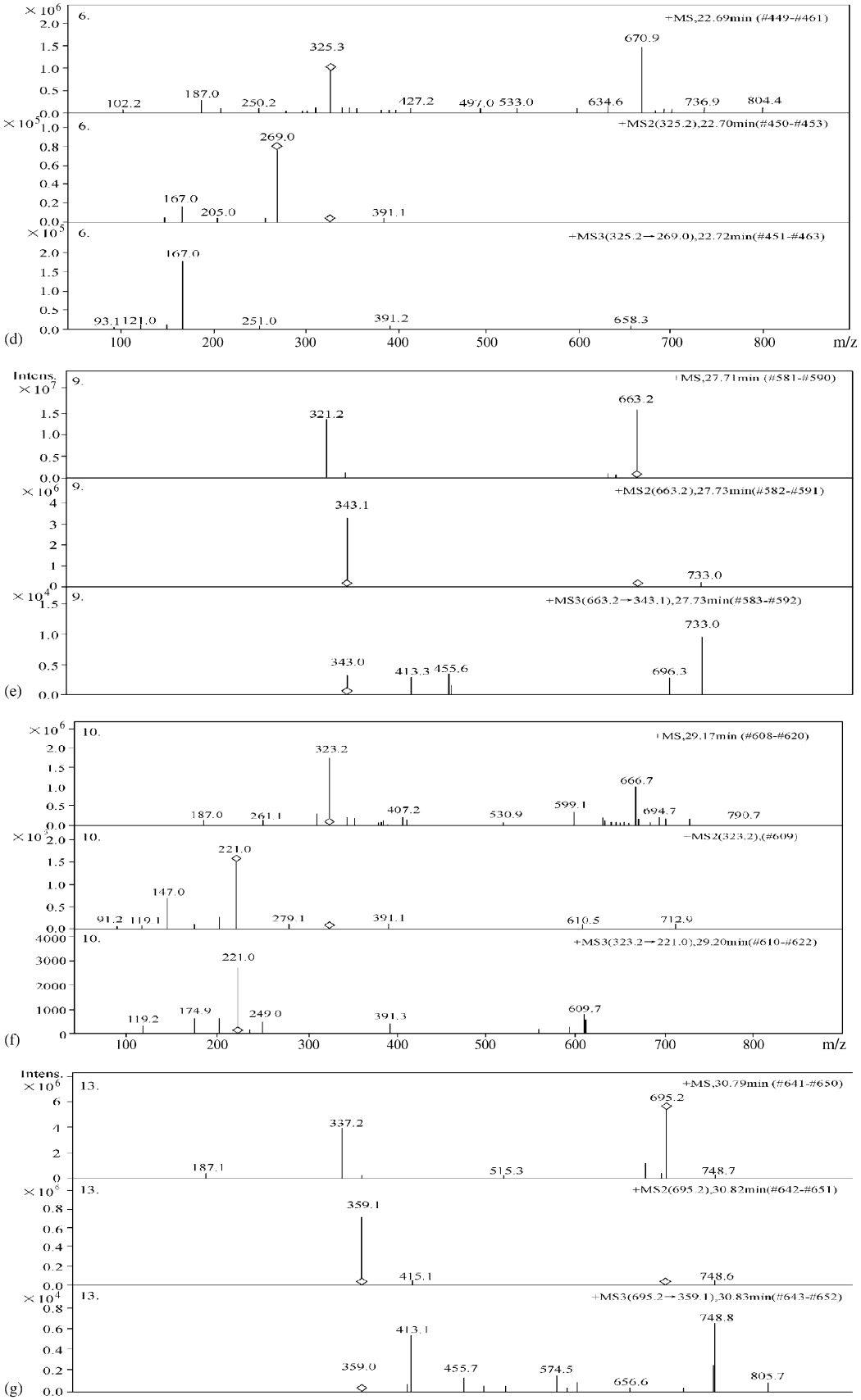


Fig. 2. (*Continued*)

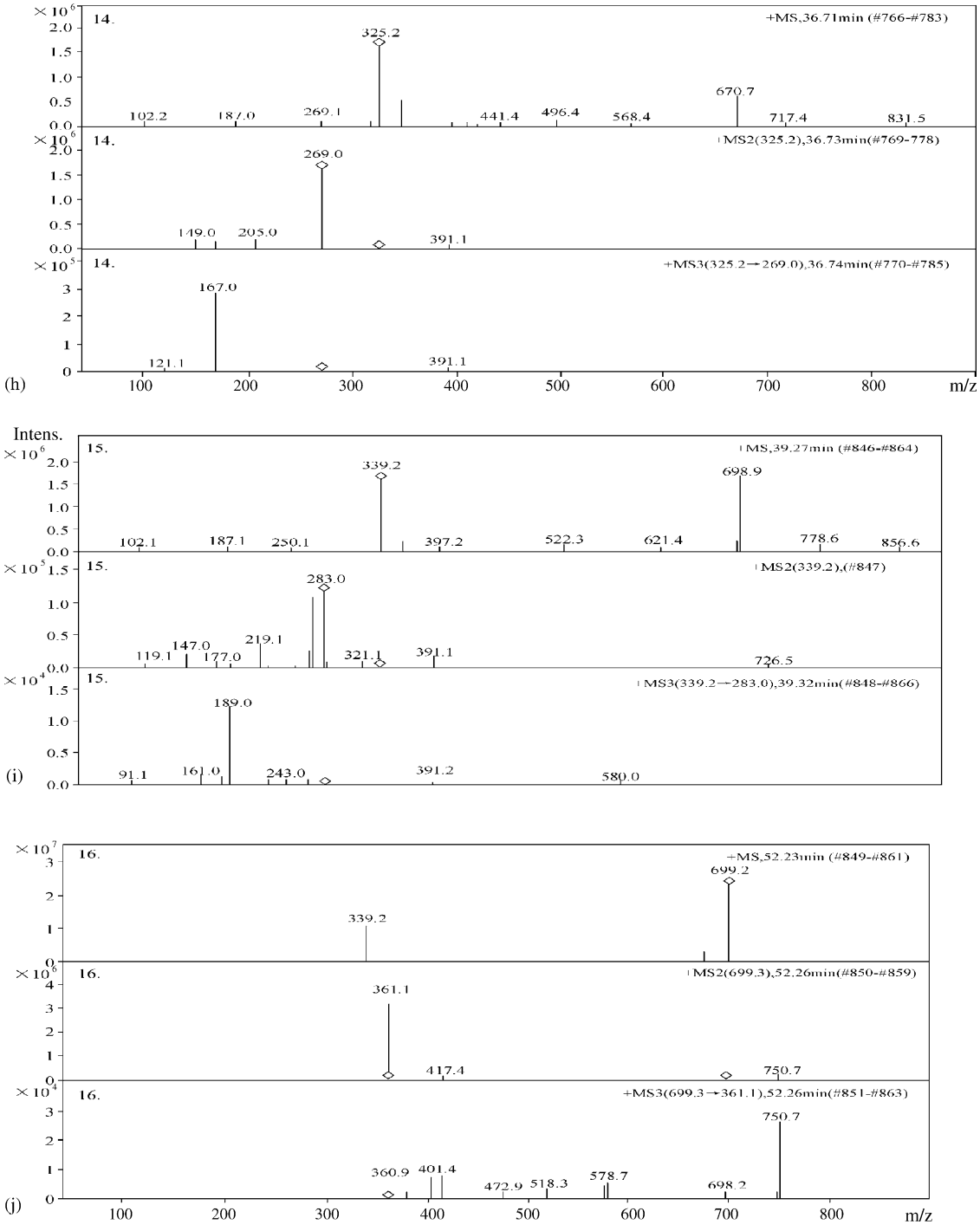


Fig. 2. (*Continued*).

are given in Table 1 and the MS, MS^2, MS^3 mass spectrum of peak numbers 1–9 and 11 are shown in Fig. 2(a)–(j).

3.4. Several examples of the structure deduced from MS data

(1) *Coumarins*: Peaks 1 and 2 in ESI(+)-MS data, $[M+H]^+$ is 187 while $[2M+K]^+$ is 391, so the molecular weight of this component is 186. This substance was deduced as psoralen or isopsoralen. Comparing with the results of the standard, we confirm peak 1 as psoralen and peak 2 as isopsoralen.

(2) *Flavones*: In ESI(+)-MS data, $[M+H]^+$ is 323 while $[2M+Na]^+$ is 667, so the molecular weight of this component is 322. *m/z* 267 is $[M-C_4H_7]^+$ and *m/z* 239 is $[M-C_4H_7-CO]^+$, so peak 3 was deduced as neobavaisoflavone.

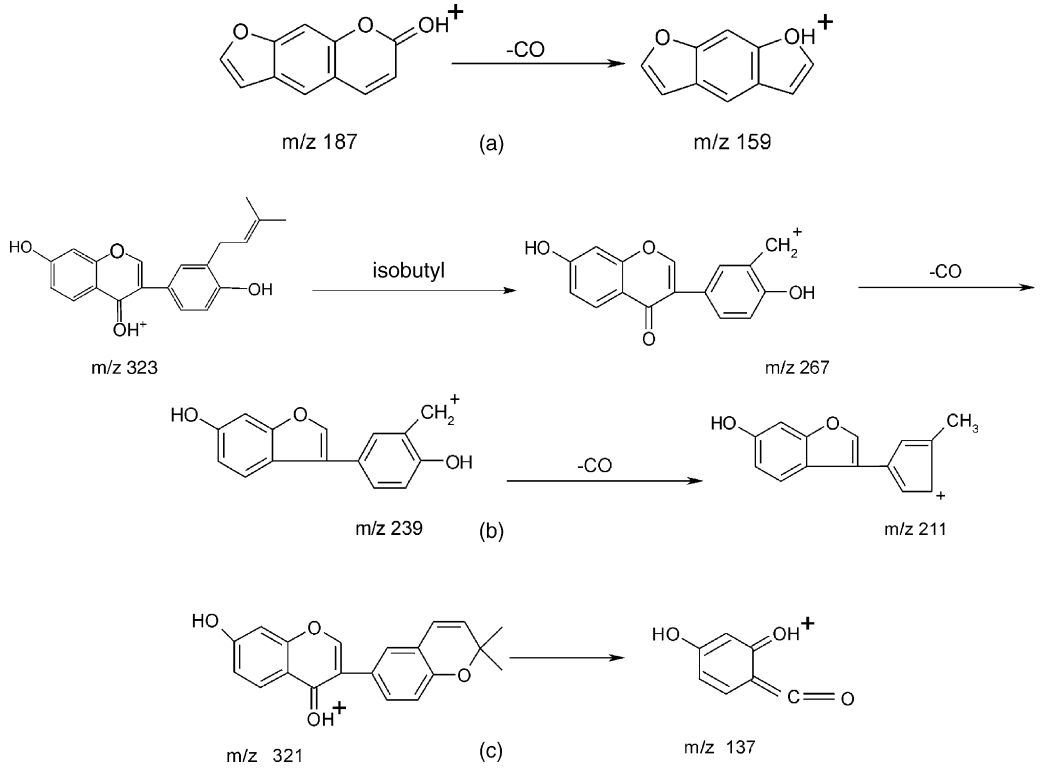


Fig. 3. Proposed MS–MS fragmentation of: (a) psoralen; (b) neobavaisoflavone; (c) corylin.

In ESI(+)-MS data, $[M + H]^+$ is 321 while $[2M + Na]^+$ is 663, so the molecular weight of this component is 320. *m/z* 303 is $[M + H - H_2O]^+$, *m/z* 137 is probably obtained from dissociative process as above, peak 5 was deduce as corylin.

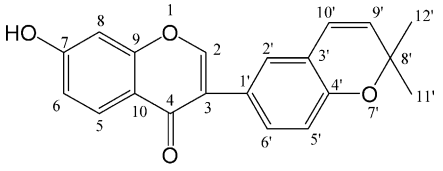
The proposed MS–MS fragmentation pathways of psoralen, neobavaisoflavone and corylin are shown in Fig. 3(a)–(c), respectively.

Peaks 10 and 12 have not been reported in reference articles, so we deduce them as new substances. Peak 10 has same fragment ion peak as neobavaisoflavone, so they probably have common kernel. Peak 12 indicates an even mass spectrum data $[M + H]^+$, so this substance contains one or more nitrogen. The fragment ion peak includes $[M + H - 150]^+$ and $[M + H - 150 - 120]^+$.

3.5. Differences in sample source

We have injected more than 20 sources of samples, including Yunnan, Burma, Anhui, Henan, Vietnam, Shanxi, etc. Though the "common peak" of each samples from different area was generally similar, the peak area ratio of the "common peak" was different significantly. The difference shows the importance of the fingerprint analysis.

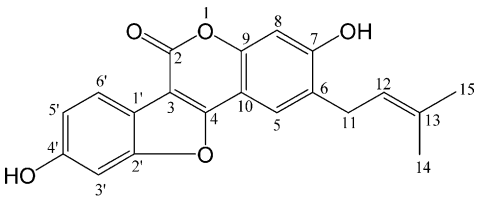
3.6. ^1H and ^{13}C NMR data



Corylin

^{1}H NMR (DMSO-d_6 + $CDCl_3$, δ, ppm): 1.42 (6H, s, 2X-CH_3); 5.70 (1H, d, J = 10.0 Hz, 9′-H); 6.38 (1H, d, J = 10.0 Hz, 10′-H); 6.76 (1H, d, J = 8.2 Hz); 6.83 (1H, d, J = 2.1 Hz, 8-H); 6.90 (1H, dd, J = 2.1, 8.8 Hz, 6-H); 7.24 (1H, d); 7.28 (1H, dd, 6′-H); 7.98 (1H, d, J = 8.7 Hz); 8.16 (1H, s, 2-H).

^{13}C NMR: 102.0 (C8); 115.0 (C6); 115.5 (C10); 116.7 (C3′); 120.5 (C3); 121.7 (C5′); 124.3 (C1′); 126.7 (C9′ or C10′); 127.0 (C5); 129.4,130.8 (C2′, C6′); 152.2 (C4′, C2); 157.4 (C9); 162.5 (C7); 174.6 (C4).



Psoralidin

^{1}H NMR (DMSO-d_6 + $CDCl_3$, δ, ppm): 1.75 (3H, s, 14-CH_3); 1.80 (3H, s, 15-CH_3); 3.34 (2H, d, J = 7.2Hz, 11-CH_2); 5.37 (1H, t, J = 7.2 Hz, 12-H); 6.92 (1H, dd, J = 2.0, 8.4Hz, 5′-H); 6.94 (1H, s, 8-H), 7.11 (1H, d, J = 2.0 Hz, 3′-H); 7.60 (1H, s, 5-H); 7.71 (1H, d, J = 8.4 Hz, 6′-H).

4. Conclusion

A HPLC method was developed for fingerprint analysis of *P. corylifolia*. The average fingerprint of 10 batches of samples from Sichuan and Henan were obtained with a standardized procedure. The fingerprint of the genuine herb showing 12 "common peaks" represents the characteristics of this herb's constituents. The results demonstrate that the method is feasible for comprehensive quality evaluation of *P. corylifolia*.

Acknowledgement

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