Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 749–759
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

# Geographical traceability of propolis by high-performance liquid-chromatography fingerprints

Jinhui Zhou, Yi Li *, Jing Zhao, Xiaofeng Xue, Liming Wu, Fang Chen

*Bee Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100093, People's Republic of China*

Received 25 June 2007; received in revised form 24 October 2007; accepted 8 November 2007

**Abstract**

A rapid fingerprint method was developed for investigating and inferring geographical origin of Chinese propolis by using high performance liquid chromatography–ultraviolet detection (HPLC–UV). 120 samples were analyzed from 17 different locations of 10 provinces of China in this study. In the HPLC chromatograms, eight major compounds were identified as flavonoids, including rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine, chrysin and galangin. Both correlation coefficient of similarity in chromatograms and relative peak areas of characteristic compounds were calculated for quantitative expression of the HPLC fingerprints. Our results revealed that the presence or absence of specific peaks and similarity evaluation in simulative mean chromatograms among different regions could efficiently identify and distinguish Chinese propolis from different geographical origins.
© 2007 Elsevier Ltd. All rights reserved.

*Keywords:* Chinese propolis; Geographical traceability; Fingerprint; HPLC; Ultrasound-assisted extraction

## 1. Introduction

Chinese propolis is a sticky, brownish resinous material that honeybees collect from leaf buds and cracks in the bark of various plants. The honeybee uses it to strengthen the borders of combs as the building material; what is more, it is regarded as the "chemical weapon" of bees against pathogen microorganisms (Wollenweber, Hausen, & Greenaway, 1990). Propolis has been used widely in folk medicine for many years because of the complex chemical compositions (Bankova, Castro, & Marcucci, 2000), and there is evidence to suggest that propolis has several medicinal properties including antibacterial (Sforcin, Fernandes, Lopes, Bankova, & Funari, 2000), antiviral (Kujumgiev et al., 1999), antitumor (Banskota et al., 2002; Murad, Calvi, Soares, Bankova, & Sforcin, 2002), anti-inflammatory (Strehl, Volpert, & Elstner, 1994), anticancer (Kimoto et al., 2001) and immunomodulatory (Bazo et al., 2002), and so on.

Flavonoids are a type of polyphenolic compounds that contain a C6–C3–C6 configuration, including flavone, flavonal, flavanone, flavanonal and isoflavone, and so on (Xiao & Lu, 1989). They have been found to be an important part of the human diet and are considered as active ingredients in propolis, especially Chinese propolis (Ng, Liu, & Wang, 2000). Research of the flavonoids in Chinese propolis has been aroused, because they have beneficial effects on health such as inhibiting the copper-catalyzed oxidation of low-density lipoprotein, inhibiting platelet clotting and arachidonate metabolism, reducing liver injury from peroxidized oil, and having cancerchemopreventative properties (Barak, Birkenfeld, Halperin, & Kalickman, 2002). The presence of eight flavonoids including rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine, chrysin and galangin can be used as a marker to differentiate propolis from other bee products. The content of eight flavonoids has been used as a parameter for propolis quality (Zhao, Li, Xue, & Cai, 2005).

Many different methods have been developed for determination of flavonoids in different plants and its relevant products, including bamboo leaves (Yu et al., 2005),

* Corresponding author. Tel.: +86 10 8259 5649; fax: +86 10 6259 4054.
*E-mail address:* wittyzhou@126.com (Y. Li).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.11.009

medicinal herb (Christopher, Perry, & Praveen, 2005; Qi et al., 2006; Wang et al., 2005), soybean pods (Stephen, Carol, Betty, & Thomas, 2003), vegetables (Ulla, Pia, & Torben, 1998), fruits (Pierre, Emile, & Alain, 1998), juices (Bronner & Beecher, 1995) and propolis (Wang, Cheng, & Xu, 2004; Zhao et al., 2005), and so on. In general, the method is used on reversed phase (RP) C18 columns, a binary solvent system containing acidified water, a polar organic solvent (acetonitrile or methanol), and ultraviolet detector (UV), photo-diode array (PDA) or mass spectrometry (MS). Flavanone glycosides are the major composition in these plants and relevant products. In propolis, the flavonoid was mainly extracted with ethanol or methanol by ultrasound-assisted, and the amount of Kaempferol, Apigenin, Chrysin and Galangin is nearly 15%.

Although there were different approaches to the analysis of flavonoids, few methods about fingerprint analysis of flavonoids have been reported for propolis in different regions of China. Fingerprint technique is an effective tool for the quality control of multi-component herbal medicines and has been widely accepted as a useful means for the evaluation and quality control of herbal materials. In the past decade, the chromatographic fingerprint established by HPLC, TLC, GC, and CE, etc. has been recognized as rapid and reliable means for the identification and qualification of herbal medicines (Xie, 2005). Some chemical fingerprint methods have been developed in various matrices including petroleum biomarkers in biota samples (Luis et al., 2007) Danshen injection (Zhang, Cui, He, Yu, & Guo, 2005), *Ginkgo biloba* extracts (Ji, Xu, Hu, & Heyden, 2005), Qianghuo (Jiang, Tao, & Shao, 2007), Fructus Psoraleae (Qiao et al., 2007) and propolis (Zhou, Zhang, Hu, & Yu, 2005; Zhu, Dou, Wei, Wang, & Lu, 2005). Among them, the HPLC fingerprint is the most important one and is widely used. In China, guidelines for the establishment of fingerprints for Chinese medicine have been officially published (State Drug Administration of China, 2000).

Propolis from the different or the same regions of China differs in their composition because of the local plants of many kinds, and it is difficult to identify the specified floral origin of certain propolis. The aim of this study was to develop a convenient HPLC–UV fingerprint method to be used for characterizing the eight major flavonoids in Chinese propolis of different regions. Then, the fingerprint model could reflect regional traceability of Chinese propolis and it will be beneficial to further confirm the quality of Chinese propolis. In this method, the propolis was extracted with ethanol and methanol by ultrasound-assisted extraction technique and created a universal process to establish HPLC–UV fingerprint, which is applicable on Chinese propolis.

## 2. Experimental

### 2.1. Reagents

Eight flavonoids including rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine, chrysin and galangin were obtained from the Sigma–Aldrich (St. Louis, MO, USA). Methanol is the HPLC grade reagents (DIMA Technology Inc., Richmond, USA). Phosphoric acid belongs to guaranteed reagent grade (Beijing chemical reagent company, Beijing, PRC). Deionized water obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Ethanol (95%, Analytical reagent grade) was purchased from Beijing chemical reagent company.

### 2.2. Preparation of standard solutions

Stock standard solutions were prepared within the range 0.1–0.8 mg/mL by dissolving eight flavonoids (rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine, chrysin and galangin) in methanol. The concentration of mixed standard solution was selected according to the level of the flavonoids expected in the propolis samples. Working mixed standard solutions were made daily by gradual dilution with methanol to the required concentration, which was based on the sensitivity of detection and the linearity range of the study. All of the standard solutions are stored at −18 °C in darkness and could be used for two months.

### 2.3. Apparatus and chromatographic conditions

The Dionex high performance liquid chromatography system consisting of a P680 quaternary pump, UVD170U UV–VIS Detector and PDA-100 detector, ASI-100 automated sample injector and thermostated column compartment was used for quantitative analysis. The data were acquired and processed by Chromeleon™ 6.70 workstation (Dionex, Sunneyvale, USA). The separation column used was a Symmetry® $C_{18}$ column, 5 μm, 4.6 × 250 mm id (Waters Part No.WAT054275, Ireland). The mobile phase was methanol/0.4%phosphoric acid (60:40) and a flow rate was 0.8 mL/min. Injection volume of solution was 10 μL. The detection wavelength was set at 280 nm.

### 2.4. Sample preparation

About 120 raw propolis samples were collected by local beekeepers (accompanied by our researcher) of different regions of China and kept at 2–8 °C. Fig. 1 shows geographical positions of the representative Chinese propolis. Voucher specimens were deposited in Bee Research Institute of Chinese Academy of Agricultural Sciences. Specifications of the samples in the present study are shown in Table 1.

The crude propolis powders were obtained after comminution and filtration (40 meshes). Powder 5.0 g was extracted by 200 mL ethanol (75%) using ultrasound-assisted extraction (Power: 100 W, Frequency: 40 kHz) for 4 h. This extraction process was repeated and extracts obtained were combined in the flask. The extracts were then isolated through a filter paper to remove macro and micro-molecular components such as minerals and bees-

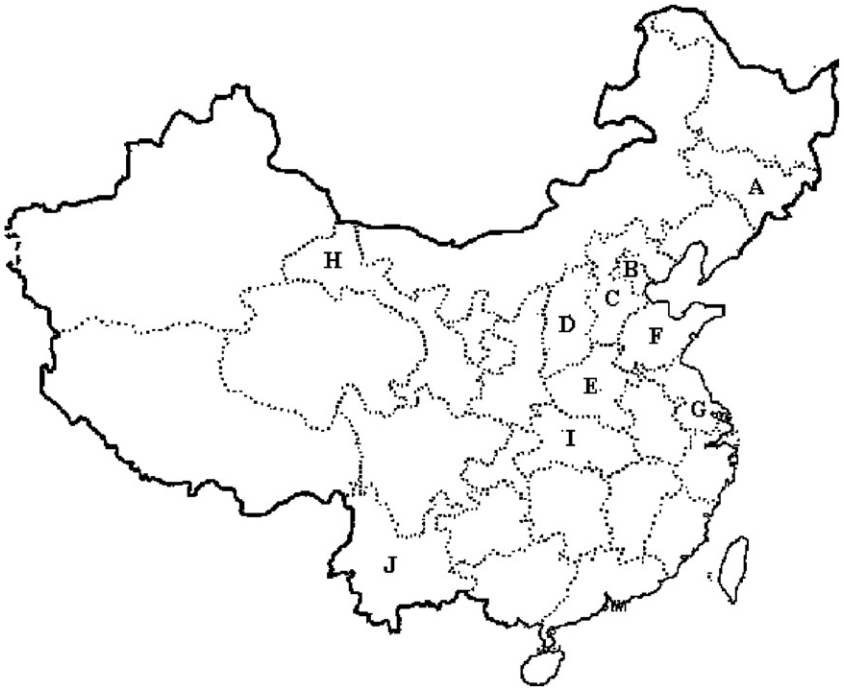


Fig. 1. Collection sites of representative Chinese propolis samples. A: Jilin province; B: Beijing; C: Hebei province; D: Shanxi province; E: Henan province; F: Shandong province; G: Jiangsu province; H: Gansu province; I: Hubei province; J: Yunnan province.

Table 1
Representative samples of Chinese raw propolis evaluated in this study

| No. | Number of samples | Growth location | Date of collection |
|---|---|---|---|
| 1 | 8 | Xiangfan, Hubei | June 2005 |
| 2 | 7 | Jingmen, Hubei | June 2005 |
| 3 | 8 | Yuan'an, Hubei | June 2005 |
| 4 | 9 | Kunming,Yunnan | July 2005 |
| 5 | 4 | Ji'an, Jilin | July 2005 |
| 6 | 7 | Jilin, Jilin | July 2005 |
| 7 | 7 | Changzhi, Shanxi | August 2005 |
| 8 | 7 | Changge, Henan | September 2005 |
| 9 | 6 | Linqu, Shandong | September 2005 |
| 10 | 7 | Yantai, Shandong | September 2005 |
| 11 | 8 | Yangzhou, Jiangsu | October 2005 |
| 12 | 7 | Rudong, Jiangsu | October 2005 |
| 13 | 7 | Northern, Gansu | September 2005 |
| 14 | 6 | Southern, Gansu | September 2005 |
| 15 | 8 | Shijiazhuang, Hebei | July 2005 |
| 16 | 8 | Mentougou, Beijing | October 2005 |
| 17 | 6 | Haidian, Beijing | October 2005 |

wax and transferred into a 250 mL pear-shape flask. Then, filtrates were evaporated to near dryness with a rotary evaporator below 35–40 °C. Residue (refined propolis) was freeze-dried and the dry powder (0.1 g) was extracted by ultrasonic technique with 20 min by adding 50 mL methanol into pear-shape flask and filtered through a 0.45 μm filter for HPLC–UV analysis.

### 2.5. Calibration curves, limits of detection and recoveries

The assay linearity was determined by analysis of six different concentrations of the standard solutions. The standard curves were obtained by plotting peak area ($y$) vs. nominal concentration ($x$ (μg/mL)) of each compound and were fitted to the linear regression $y = ax + b$. Concentrations of these marker substances in samples were calculated from this regression analysis. LOD was defined as the quantity of compound required for an S/N of three. The accuracy tests were carried out by spiking known contents of mixed standard solution into known concentration of propolis samples and assessed by analyzing three different spiking concentrations of analytes in triplicate replications. The percent recoveries for the analytes were defined as mean (found concentration/actual concentration) × 100%.

### 2.6. Method validation

The injection precision was determined by replicate injection of the same sample solution for five times in a day. The sample stability test precision was determined with one sample during seven days. The repeatability was assessed by analyzing five independently prepared samples of propolis samples. During this period, the solution was stored at room temperature.

### 2.7. Data analysis

Data analysis was performed by professional fingerprint software named "*Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine* (Version 2004A)", which is recommended by state food and drug administration (SFDA) of China and mainly used in the similarity analysis of chromatographic and spectral

patterns (Liang, Xie, & Chan, 2004; Wang, Xiao, Liang, & Bi, 2002;). In the present study, this software has been used to synchronize the chromatographic peaks and to calculate the correlation coefficients between entire chromatographic profiles, as well as to compute and generate the mean chromatogram as a representative standard fingerprint chromatogram for a group of chromatograms. Besides, Considering the characteristics of fingerprints, the relative retention time (RRT) and relative peak area (RPA) of main peaks (>1% of total peak area) detected were used to evaluate the quality of fingerprints. The relative retention time (RRT) and relative peak area (RPA) of each characteristic peak were calculated for quantitative expression of the chemical properties in the chromatographic pattern. The quantitative method is only one of purposes of HPLC fingerprint. The parameters evaluated and validation aspects are different from general assaying methods (Ji, Xu, Hu, & Heyden, 2005).

## 3. Results and discussion

### 3.1. Selection of chromatographic conditions

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of adjacent peaks. Different mobile phase compositions were optimized: formic acid, acetic acid and phosphoric acid were added to the aqueous phase of mobile phase to enhance the resolution, restrain the ionization of flavonoids and eliminate the peak tailing of target compounds. As a result, mobile phases containing phosphoric acid were selected. Mobile phase with methanol has a satisfactory resolution and stable baseline. To acquire better selectivity and higher efficiency, eluent pH over the range of 2.0–5.0 was tested and different concentrations of phosphoric acid in the aqueous phase were also investigated. In the end, the mobile phase consisting of methanol and 0.4% phosphoric acid (pH 3.0) were chosen for the determination of eight flavonoids in Chinese propolis.

On the ultraviolet spectra with chromatograms of HPLC–UV of eight flavonoids in propolis and reference standards, maximum absorbance values around 280 nm and 350 nm were observed. More detectable peaks could be obtained and the baseline was well improved around 280 nm at which the better characterization of flavonoids can be attributed. Hence, characteristic chromatographic patterns were obtained by using 280 nm as the detection wavelength.

### 3.2. Optimization of extraction procedure

In order to obtain optimal extraction efficiency, extraction solvents and extraction time were investigated. Various solvents including methanol, ethanol, glycerol, petroleum ether, chloroform, ethyl acetate and acetone were tested for the extraction of raw propolis sample. Ethanol was particularly suitable to obtain dewaxed propolis extracts (Pietta, Gardana, & Pietta, 2002). Aqueous ethanol was the preferred choice of extraction solvent as a variety of compounds with different polarity. In this study, 75% aqueous methanol (v/v) was chosen as the extraction solvent not only because the raw propolis could be efficiently dissolved but also the bee wax could be remarkably separated from the raw propolis solutions. The ultrasound (4 h), reflux (4 h), and Soxhlet extraction (4 h) were compared in parallel experiments using 75% ethanol as the solvent. The ultrasound-assisted extraction method (4 h) showed the greatest extraction ability. The extraction of flavonoids in refined propolis was performed by using methanol with ultrasound-assisted extraction because of the satisfactory recovery and the accordance of mobile phase system.

### 3.3. Calibration curves, limits of detection and recoveries

The regression equation, correlation coefficient, linear response range, and detection limit of rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine, chrysin and galangin are $y = 1.6067x-0.5424$, $r^2 = 0.9992$, 2 – 500 mg/L, 0.2 mg/g; $y = 0.4922x + 2.8082$, $r^2 = 0.9991$, 1 – 500 mg/L, 0.1 mg/g; $y = 2.3654x + 25.555$, $r^2 = 0.9991$, 2 – 500 mg/L, 0.2 mg/g; $y = 2.7442x + 4.8181$, $r^2 = 0.9999$, 1 – 500 mg/L, 0.1 mg/g; $y = 3.0213x + 12.617$, $r^2 = 0.9992$, 1 – 500 mg/L, 0.1 mg/g; $y = 0.9393x - 2.1998$, $r^2 = 0.9992$, 2 – 500 mg/L, 0.2 mg/g; $y = 3.6745x - 19.713$, $r^2 = 0.9993$, 1 – 1000 mg/L, 0.1 mg/g and $y = 1.5719x - 1.7221$, $r^2 = 0.9999$, 2 – 1000 mg/L, 0.2 mg/g respectively. These results, through linear regression analysis, showed a good linear relationship between the peak area ($y$) and the concentration ($x$ (μg/mL)). The recoveries of the eight flavonoids were between 89.4 and 96.3% and the RSDs were less than 4%.

### 3.4. HPLC quantitative analysis

All raw propolis samples were analyzed using the established extraction method under the above HPLC conditions. Each sample was analyzed in triplicate to determine the mean contents of eight major flavonoids in Chinese propolis samples. The results showed that no significant difference was found for mean contents of eight major flavonoids of the studied samples among different provinces of China. The content of 8 major flavonoids in the same province ranges (w/w) were 0.079–0.728% (Rutin), 0.189–0.745% (Myricetin), 0.027–0.542% (Quercetin), 0.171–0.736% (Kaempferol), 0.132–0.474% (Apigenin), 1.024–3.956% (Pinocembrine), 0.338–3.597% (Chrysin) and 0.871–7.057% (Galangin), respectively. The significant variability might be caused by different and mixed botanical origin in various samples of the same province.

### 3.5. Fingerprint analysis of Chinese propolis

Propolis has been used widely in folk medicine for many years, and there is substantial evidence to indicate that

propolis has antiseptic, antifungal, antibacterial, antiviral, anti-inflammatory and antioxidant properties (Castaldo & Capasso 2002). Current applications of propolis include over-the-counter preparations for cold syndrome (upper respiratory tract infections, common cold, flu-like infection) as well as dermatological preparations useful in wound healing, treatment of boils, acne, herpes simplex and genitalis, and neurodermatitis (Banskota, Tezuka, & Kadota, 2001; Volpi & Bergonzini, 2006).

There are various plant species in different provinces and regions of China because of vast territory and abundant resources in China. Hence, a piece of propolis usually comes from various plants and it is difficult to ascertain the detail botanical origin. The geographical traceability of Chinese propolis became more and more necessary. About 120 samples of raw propolis collected from different regions of China were analyzed with the above extraction method and HPLC condition. The results indicated that their chromatographic patterns were generally consistent with its origin although the quantity and absorption intensity of peaks was different.

The peaks in HPLC chromatograms were identified based on retention time and UV adsorption spectra comparison. In these HPLC fingerprints, 29 peaks were chosen in Chinese propolis samples and were presented in a relatively high content (Fig. 2). Peak 19 was assigned as the reference peak because it was the highest peak and had a moderate retention time in the Chinese propolis chromatograms. The HPLC fingerprints of Chinese propolis samples were further quantitatively expressed in terms of RRT and RPA. The RRT and RPA of each characteristic peak with respect to the reference peak were calculated (Table 2). The RRT and RPA data of

Table 2
The relative retention time (RRT) and relative peak area (RPA) of characteristic peaks in Chinese propolis samples

| Peak no. | Compounds | RRT[a] | RPA[b] |
|---|---|---|---|
| 1 | Rutin | 0.136 | 0.205 ± 0.185 |
| 2 | Unknown | 0.147 | 1.071 ± 0.436 |
| 3 | Myricetin | 0.192 | 0.197 ± 0.195 |
| 4 | Unknown | 0.225 | 0.103 ± 0.061 |
| 5 | Quercetin | 0.269 | 0.232 ± 0.216 |
| 6 | Unknown | 0.323 | 0.296 ± 0.127 |
| 7 | Kaempferol | 0.422 | 0.144 ± 0.071 |
| 8 | Apigenin | 0.449 | 0.117 ± 0.095 |
| 9 | Unknown | 0.488 | 0.058 ± 0.041 |
| 10 | Unknown | 0.511 | 0.134 ± 0.131 |
| 11 | Unknown | 0.615 | 0.023 ± 0.021 |
| 12 | Unknown | 0.701 | 0.049 ± 0.036 |
| 13 | Unknown | 0.739 | 0.031 ± 0.017 |
| 14 | Unknown | 0.768 | 0.034 ± 0.025 |
| 15 | Unknown | 0.786 | 0.305 ± 0.030 |
| 16 | Pinocembrine | 0.812 | 0.885 ± 0.317 |
| 17 | Unknown | 0.827 | 0.094 ± 0.059 |
| 18 | Unknown | 0.860 | 0.049 ± 0.037 |
| 19 | Chrysin | 1.000 | 1.00 ± 0.00 |
| 20 | Unknown | 1.081 | 0.065 ± 0.035 |
| 21 | Unknown | 1.127 | 0.059 ± 0.057 |
| 22 | Unknown | 1.190 | 0.024 ± 0.023 |
| 23 | Galangin | 1.272 | 0.517 ± 0.216 |
| 24 | Unknown | 1.337 | 0.137 ± 0.081 |
| 25 | Unknown | 1.372 | 0.274 ± 0.258 |
| 26 | Unknown | 1.489 | 0.033 ± 0.029 |
| 27 | Unknown | 1.721 | 0.066 ± 0.061 |
| 28 | Unknown | 1.938 | 0.046 ± 0.043 |
| 29 | Unknown | 2.140 | 0.016 ± 0.015 |

[a] The average relative retention time of each characteristic peak to the reference peak (compound 19).
[b] The relative peak area of each characteristic peak to the reference peak (compound 19). The value is mean ± SD.

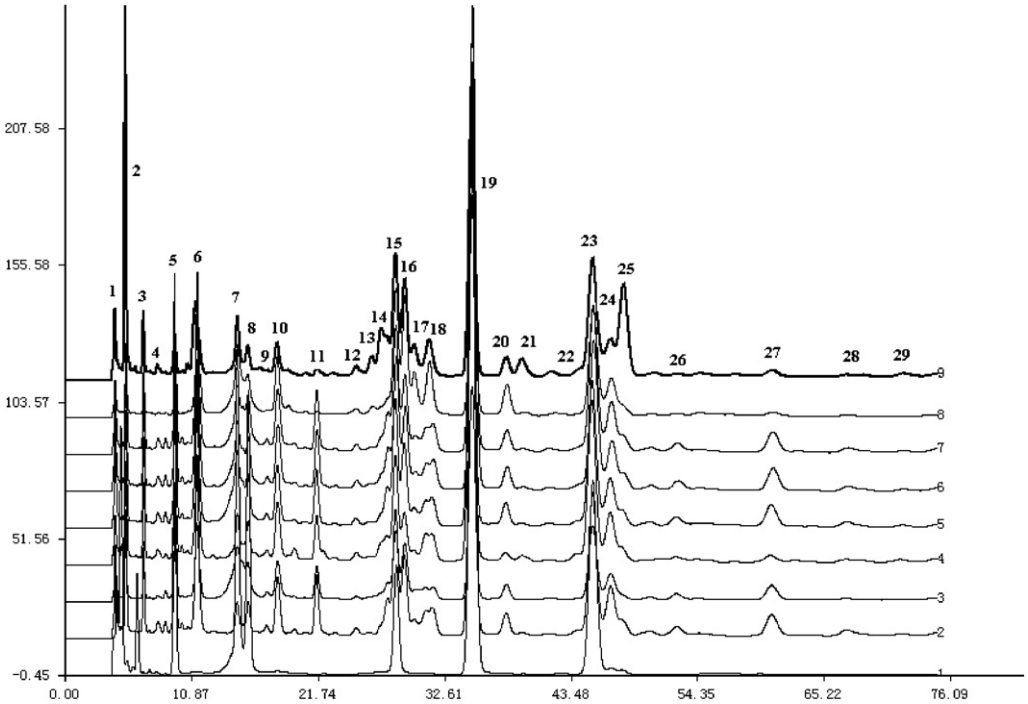


Fig. 2. Representative HPLC chromatogram fingerprint of Chinese propolis.

the characteristic peaks may reflect the quantitative expression of the present HPLC fingerprint of Chinese propolis.

### 3.6. Precision, repeatability and stability

The relative standard deviation (RSD) of RRT and RPA found were not exceeding 0.27% and 2.96% for injection precision, respectively. The relative standard deviation of RRT and RPA were below 0.39% and 3.75% for sample stability test, respectively. The RSD of RRT and RPA found were less than 0.58 and 2.89% for five independent samples respectively. The similarity of these results with those from the repeatability and the injection precision indicate that the sample remained stable when the solution was stored at room temperature.

### 3.7. HPLC fingerprint comparison of Chinese propolis in different provinces and regions

The differences of Chinese propolis from the different provinces and regions were further studied. The 120 samples were collected from 17 different locations in northeast, north China, central plain, northwest, east China and central-south China of China. All these samples were evaluated by the established HPLC fingerprint test procedure.

Chromatograms of adjacent provinces or various regions of the same province were shown in a sole fingerprint for legible observation and comparison. The similarity of each province propolis samples to their simulative mean chromatogram ranged from $0.793 \pm 0.042$ to $0.962 \pm 0.023$ (mean± SD, Table 3). The farther the geographical location is, the less the similarities of simulative mean chromatogram to that of other provinces propolis samples gets. More samples were needed to obtain a more representative population.

The similarity of each Beijing and Hebei province sample chromatogram to their simulative mean chromatogram were $0.951 \pm 0.019$ ($n = 14$) and $0.929 \pm 0.025$ ($n = 8$), respectively. They have found highly resembling to each other (Fig. 3a). The similarity of the simulative mean chromatogram of Beijing sample to that of Hebei province sample was $0.916 \pm 0.037$. The simulative mean chromatogram demonstrated that the chemical components in Beijing and Hebei province sample were slight differences. There are peak 9, 22 and 28 in the simulative mean chromatogram of Hebei province sample, but none in Beijing. Moreover, in Beijing, there are peak 4 and 26 in the simulative mean chromatogram of Haidian district, but none in Mentougou district. The results showed that the relationship of two propolis species of Beijing and Hebei province was very close in term of chemotaxonomy. The main reason lies in the nearest location between Beijing and Hebei province.

For Jilin province samples, the similarity of each chromatogram to their simulative mean chromatogram was $0.846 \pm 0.028$ ($n = 10$). There are peak 17 and 24 in the simulative mean chromatogram of Jilin city, but none in Jian city. However, the simulative mean chromatogram

Table 3
Similarity comparison of chromatographic patterns of Chinese propolis in different regions of China

| Sample from different location | Jilin | Beijing | Hebei | Shanxi | Shandong | Henan | Gansu | Jiangsu | Yunnan | Hubei |
|---|---|---|---|---|---|---|---|---|---|---|
| Jilin | $0.846 \pm 0.028^{a}$ | $0.762 \pm 0.025^{b}$ | $0.694 \pm 0.031^{b}$ | $0.527 \pm 0.024^{b}$ | $0.357 \pm 0.011^{b}$ | $0.247 \pm 0.010^{b}$ | $0.785 \pm 0.026^{b}$ | $0.533 \pm 0.021^{b}$ | $0.485 \pm 0.013^{b}$ | $0.473 \pm 0.018^{b}$ |
| Beijing | | $0.951 \pm 0.019^{a}$ | $0.916 \pm 0.037^{b}$ | $0.709 \pm 0.036^{b}$ | $0.493 \pm 0.023^{b}$ | $0.371 \pm 0.014^{b}$ | $0.766 \pm 0.032^{b}$ | $0.602 \pm 0.025^{b}$ | $0.772 \pm 0.017^{b}$ | $0.616 \pm 0.029^{b}$ |
| Hebei | | | $0.929 \pm 0.025^{a}$ | $0.811 \pm 0.043^{b}$ | $0.681 \pm 0.037^{b}$ | $0.638 \pm 0.022^{b}$ | $0.695 \pm 0.029^{b}$ | $0.751 \pm 0.028^{b}$ | $0.793 \pm 0.031^{b}$ | $0.609 \pm 0.016^{b}$ |
| Shanxi | | | | $0.874 \pm 0.031^{a}$ | $0.785 \pm 0.032^{b}$ | $0.843 \pm 0.026^{b}$ | $0.655 \pm 0.031^{b}$ | $0.915 \pm 0.039^{b}$ | $0.651 \pm 0.024^{b}$ | $0.572 \pm 0.015^{b}$ |
| Shandong | | | | | $0.928 \pm 0.037^{a}$ | $0.641 \pm 0.030^{b}$ | $0.668 \pm 0.029^{b}$ | $0.919 \pm 0.033^{b}$ | $0.636 \pm 0.018^{b}$ | $0.602 \pm 0.007^{b}$ |
| Henan | | | | | | $0.940 \pm 0.031^{a}$ | $0.874 \pm 0.042^{b}$ | $0.619 \pm 0.015^{b}$ | $0.513 \pm 0.021^{b}$ | $0.896 \pm 0.014^{b}$ |
| Gansu | | | | | | | $0.901 \pm 0.036^{a}$ | $0.611 \pm 0.011^{b}$ | $0.244 \pm 0.009^{b}$ | $0.366 \pm 0.006^{b}$ |
| Jiangsu | | | | | | | | $0.952 \pm 0.044^{a}$ | $0.528 \pm 0.017^{b}$ | $0.538 \pm 0.011^{b}$ |
| Yunnan | | | | | | | | | $0.793 \pm 0.042^{a}$ | $0.684 \pm 0.021^{b}$ |
| Hubei | | | | | | | | | | $0.962 \pm 0.023^{a}$ |

[a] The similarity of each chromatogram to their simulative mean chromatogram, mean ± SD.
[b] The similarity between simulative mean chromatograms, mean ± SD.

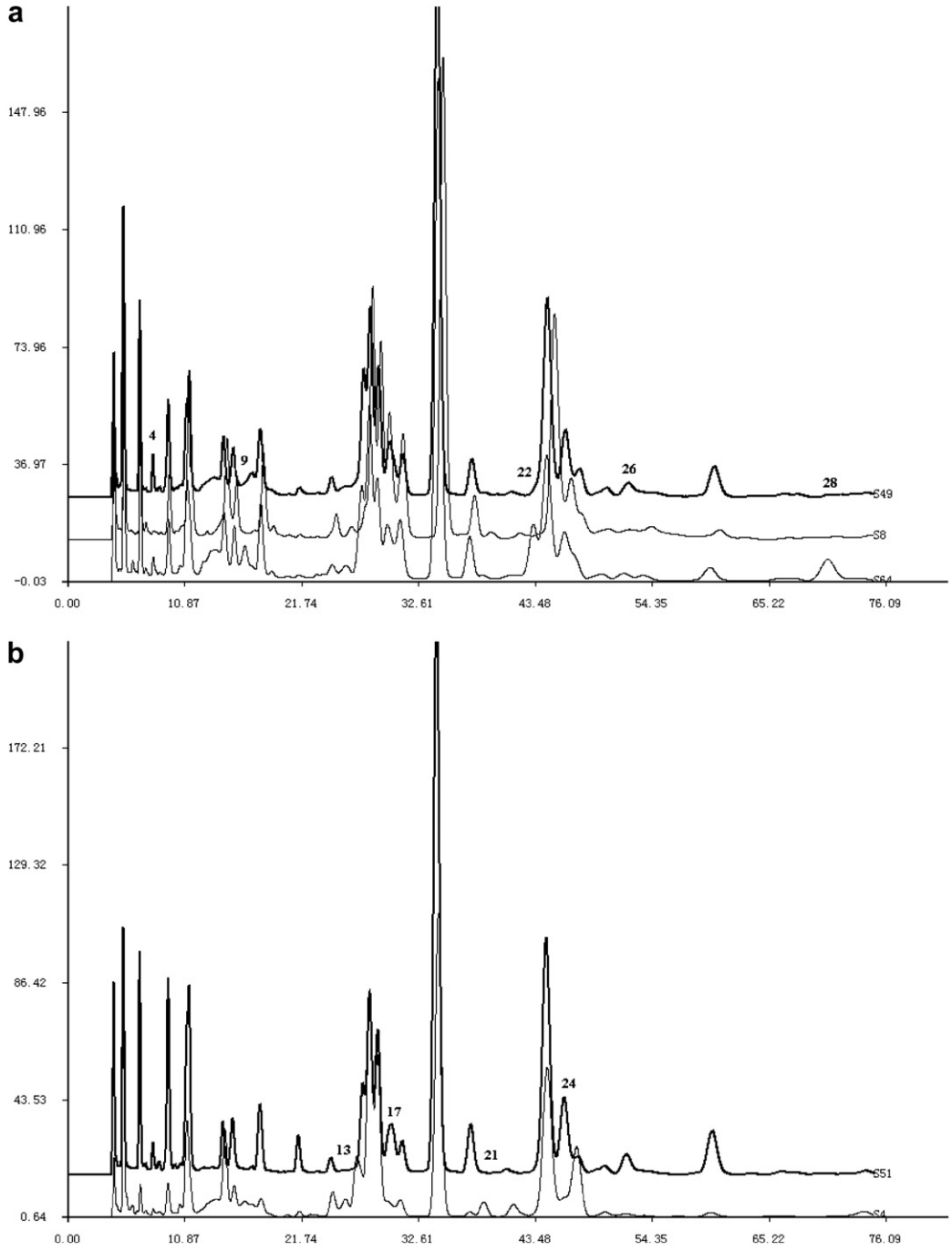


Fig. 3. Representative HPLC chromatogram fingerprint of Chinese propolis (a) Beijing and Hebei; (b) Jilin; (c) Hubei and Yunnan; (d) Shanxi and Henan; (e) Shandong and Jiangsu; (f) Gansu.

of Jian city sample has peak 13 and 21, but they were not found in Jilin city sample (Fig. 3b). Besides, the chromatogram of Jilin province sample showed significant differences from that of other province samples. The similarities of each Jilin province sample chromatogram to Henan, Shandong, Yunnan and Hubei provinces were only $0.247 \pm 0.010$, $0.357 \pm 0.011$, $0.485 \pm 0.013$ and $0.473 \pm 0.018$, respectively. The almost northernmost location of Jilin provinces resulted in the low similarity to other places.

For Hubei province and Yunnan province propolis samples, the similarity of each Hubei province and Yunnan province sample chromatogram to their simulative mean chromatogram were $0.962 \pm 0.023$ ($n = 23$) and $0.793 \pm 0.042$ ($n = 9$), respectively. The similarity of the simulative mean chromatogram of Hubei sample to that of Yunnan province sample was only $0.684 \pm 0.021$. There are peak 11, 22 and 26 in the Hubei province propolis samples, but absence in Yunnan province (Fig. 3c). In contrast, peak 21 was found in Yunnan province samples, but absence in

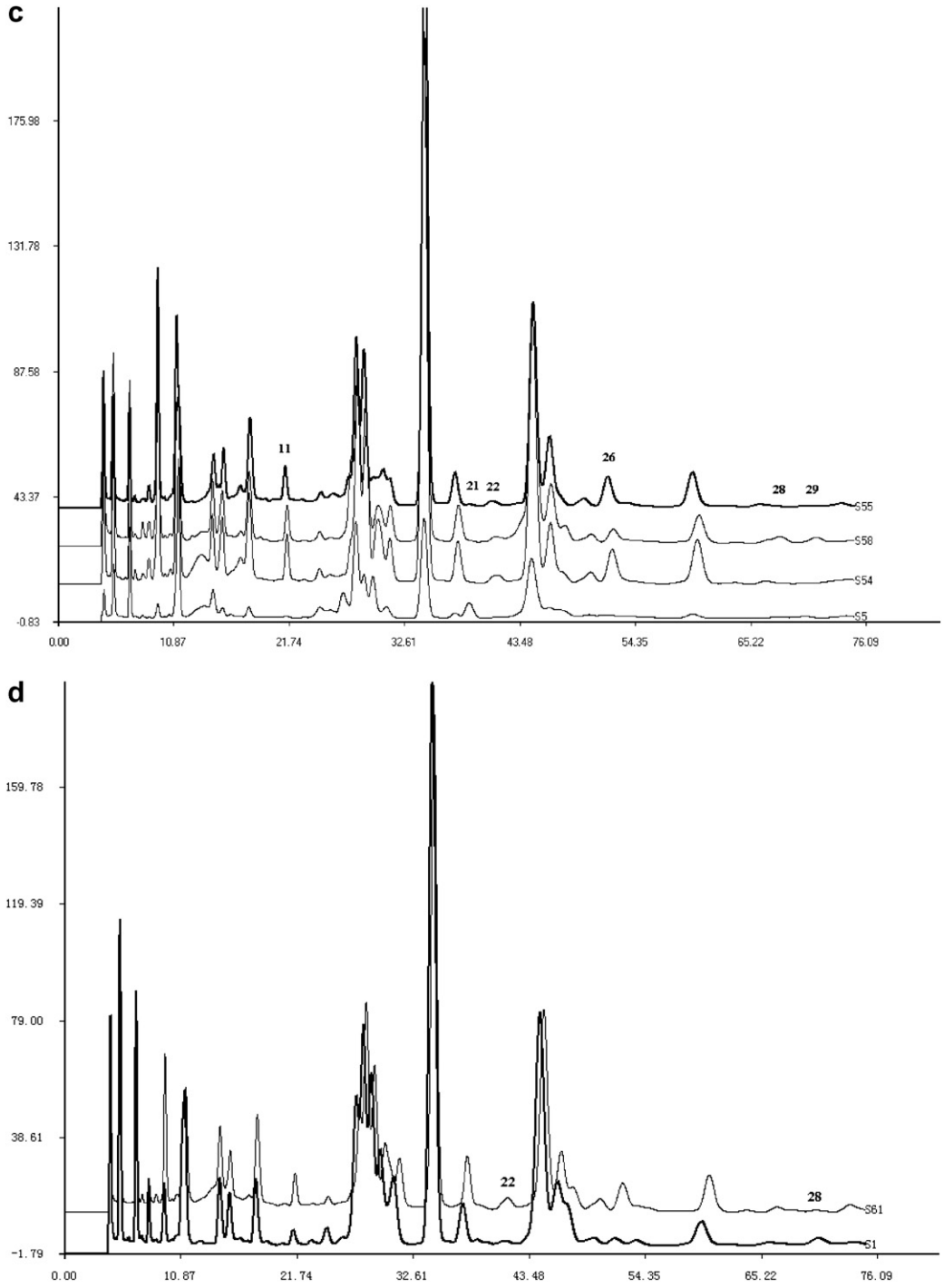


Fig. 3 (*continued*)

Hubei province. Besides, peak 28 and 29 were found in Yuanan city of Hubei province, but absence in Jingmen and Xiangfan cities. The similarity of the simulative mean chromatogram of Hubei province sample to that of Jilin and Gansu province sample was only $0.473 \pm 0.018$ and $0.366 \pm 0.006$. The similarity of the simulative mean chromatogram of Yunnan province sample to that of Jilin and Gansu province sample was only $0.485 \pm 0.013$ and $0.244 \pm 0.009$. The farthest geographical distance between South China (Hubei or Yunnan province) and North China (Jilin or Gansu) results in the low similarity. Comparing with other provinces, Yunnan province has more kinds of plants which are main reason of the comparatively poor similarity.

For Henan province and Shanxi province propolis samples, the similarity of each Henan province and Shanxi province sample chromatogram to their simulative mean chromatogram were $0.940 \pm 0.031$ ($n = 7$) and $0.874 \pm$

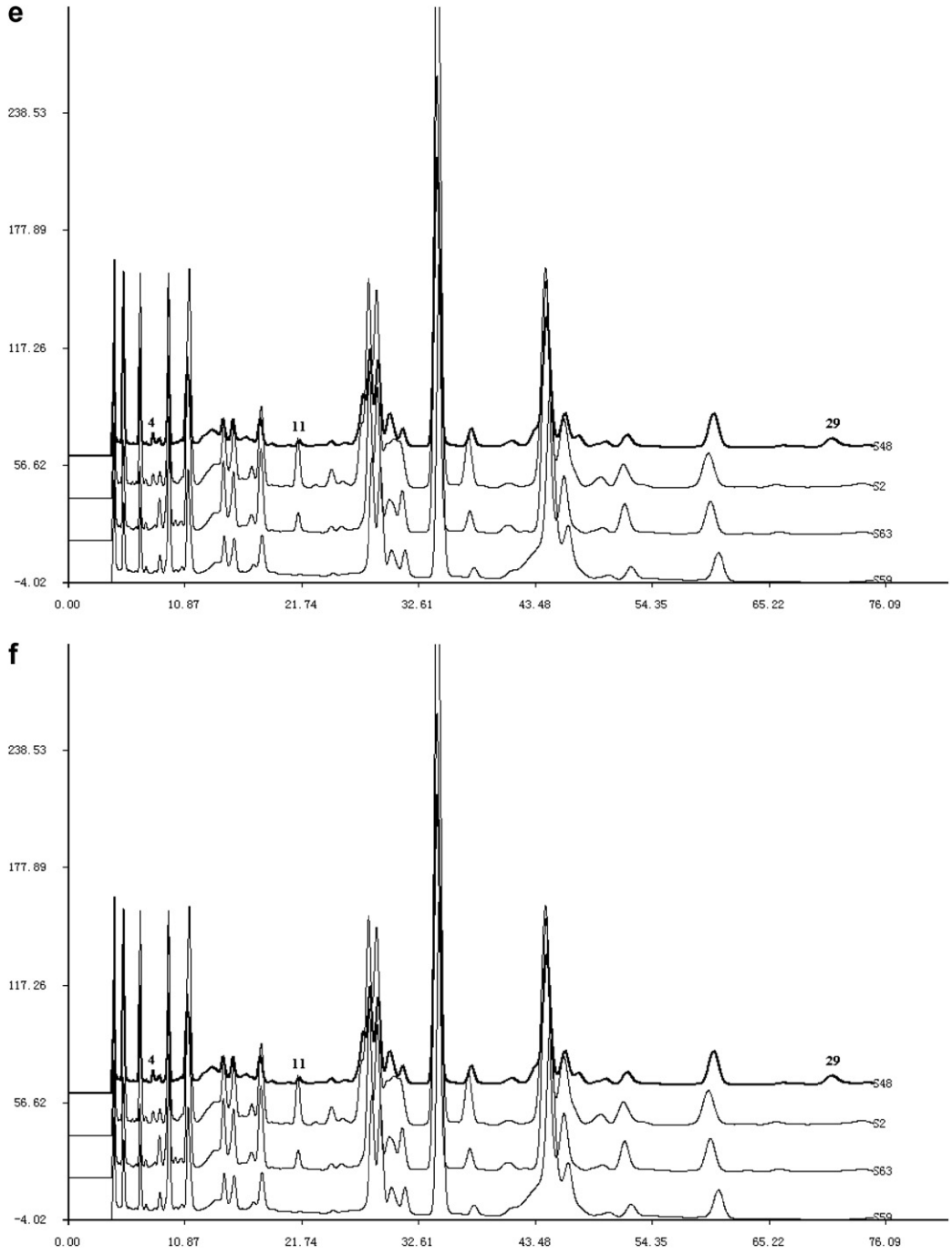


Fig. 3 (*continued*)

0.031 ($n = 7$), respectively. The similarity of the simulative mean chromatogram of Hubei sample to that of Yunnan province sample was $0.873 \pm 0.026$. The contents of compound 22 (peak 22) in propolis samples of Henna province were remarkably higher than those in propolis samples of Shanxi province. However, the contents of peak 28 in propolis samples of Henan province are obviously lower than those in propolis samples of Shanxi province. The chromatograms of Henan and Shanxi province propolis samples were found highly resembling to each other (Fig. 3d) because they are the adjacent province which possessed the similar plant species.

Shandong province and Jiangsu province has rich plant resources. The similarity of each Shandong province and Jiangsu province sample chromatogram to their simulative mean chromatogram were $0.928 \pm 0.037$ ($n = 14$) and $0.952 \pm 0.044$ ($n = 15$), respectively. The similarity of the simulative mean chromatogram of Shandong province sample to that of Jiangsu province sample was $0.919 \pm 0.033$. Fig. 3e showed that (1) there is peak four in Shandong province, absence in Jiangsu province; (2) there is peak 11 in Shandong province and Yangzhou city of Jiangsu province, absence in Rudong city of Jiangsu province; (3) there is peak 29 in Yantai city of Shandong

province, but it is not found in other cities of Shandong province and Jiangsu province. The results indicated that there was resemblance in terms of chemical constituents of samples of Shandong province and Jiangsu province. Nevertheless, it was easy to identify them based on the peak 4, 11 and 29 of the above mentioned.

The unique climate of arid or moist, warm or cold in turn resulted in the specific botanical subspecies of Gansu province in northwest China. The similarity of each chromatogram to their simulative mean chromatogram was $0.901 \pm 0.036$ ($n = 13$). There is peak 26 in samples of north of Gansu province, while absent in south of Gansu province (Fig. 3f). In addition, it is obvious that the content of compound 23 (peak 23) were drastically lower than that compound 24 (peak 24) in samples of north of Gansu province. In contrast, the content of compound 23 (peak 23) were remarkably higher than that compound 24 (peak 24) in samples of north of Gansu province. The results indicated that the main chemical components in samples of north and south of Gansu province were quite similar.

## 4. Conclusion

In this study, the HPLC fingerprint of Chinese propolis was successfully established for the first time. About 120 propolis samples from different provinces of China were identified and distinguished by the chromatographic fingerprint in combination with similarity analysis. The results of our study showed that no significant difference was found for mean contents of eight major flavonoids among the studied samples in different provinces of China. For the similarity analysis by using "*Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine*", the further the geographical location is, the less the similarities of simulative mean chromatogram to that of other provinces propolis samples gets. The presence or absence of some peaks in simulative mean chromatograms depended on the geographical origin character of Chinese propolis.

The fingerprinting analysis combining similarity evaluation is a novel, valid and rapid method for the geographical origin of Chinese propolis. The advantage of using chromatographic profiles for geographical origin of propolis is that it is often unnecessary to know the individual components that make up the fingerprint (Kelly, 2001). Thus; this presents itself as a highly rapid and efficient process for assessment. The fingerprint method established by this study could be applied to the propolis of other countries and herbal plants for the assessment of geographical origin.

## References


Bankova, V. S., Castro, S. L., & Marcucci, M. C. (2000). Propolis: Recent advances in chemistry and plant origin. *Apidologie, 31*, 3–15.

Banskota, A. H., Nagaoka, T., Sumioka, L. Y., Tezuka, Y., Awale, S., & Midorikawa, K. (2002). Antiproliferative activity of the Netherlands propolis and its active principles in cancer cells lines. *Journal of Ethnopharmacology, 80*, 67–73.

Banskota, A. H., Tezuka, Y., & Kadota, S. (2001). Recent progress in pharmacological research of propolis. *Phytotherapy Reserach, 15*, 561–571.

Barak, V., Birkenfeld, S., Halperin, T., & Kalickman, I. (2002). The effect of herbal remedies on the production of human inflammatory and anti-inflammatory cytokines. *The Isreal Medical Association Journal, 4*(Suppl. 11), 919–922.

Bazo, A. P., Rodrigues, M. A., Sforcin, J. M., Camargo, J. L., Ribeiro, L. R., & Salvadori, D. M. (2002). Protective action of propolis on the rat colon. *Carcinogenesis, Teratogenesis, Carcinogenesis and Mutagenesis, 22*, 183–194.

Bronner, W. E., & Beecher, G. R. (1995). Extraction and measurement of prominent flavonoids in orange and grapefruit juice concentrates. *Journal of Chromatography A, 705*, 247–256.

Castaldo, S., & Capasso, F. (2002). Propolis an old remedy used in modern medicine. *Fitoterapia, 73*, S1–S6.

Christopher, R. H., Perry, A. M., & Praveen, K. S. (2005). Identification and quantification of eight flavones in root and shoot tissues of the medicinal plant Huang-qin (*Scutellaria baicalensis* Georgi) using high-performance liquid chromatography with diode array and mass spectrometric detection. *Journal of Chromatography A, 1062*, 199–207.

Ji, Y. B., Xu, Q. S., Hu, Y. Z., & Heyden, Y. V. (2005). Development, optimization and validation of a fingerprint of Ginkgo biloba extracts by high-performance liquid chromatography. *Journal of Chromatography A, 1066*, 97–104.

Jiang, F. Q., Tao, Y. D., & Shao, Y. (2007). Fingerprinting quality control of Qianghuo by high-performance liquid chromatography-photodiode array detection. *Journal of Ethnopharmacology, 111*, 265–270.

Kelly, L. (2001). Herbal medic ines-analytical challenges faced by a regulatory laboratory. In International symposium on quality of TCM with chromatographic fingerprint, Vol. 259, p. 57. Guangzhou, China.

Kimoto, T., Aga, M., Hino, K., Koya-Miyata, S., Yamamoto, Y., & Micallef, M. J. (2001). Apoptosis of human leukemia cells induced by artepillin C, an active ingredient of Brazilian propolis. *Anticancer Research, 21*, 221–228.

Kujumgiev, A., Tsvetkova, I., Serkedjieva, Y., Bankova, V., Christov, R., & Popov, S. (1999). Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *Journal of Ethnopharmacology, 64*, 235–240.

Liang, Y. Z., Xie, P. S., & Chan, K. (2004). Quality control of herbal medicines. *Journal of Chromatography A, 812*, 53–70.

Luis, B., Miren, D., Nestor, E., Patricia, N., Aresatz, U., & Olatz, Z. (2007). Chemical fingerprinting of petroleum biomarkers in biota samples using retention-time locking chromatography and multivariate analysis. *Journal of Chromatography A, 1157*, 369–375.

Murad, J. M., Calvi, S. A., Soares, A. M. V. C., Bankova, V., & Sforcin, J. M. (2002). Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against paracoccidioides brasiliensis. *Journal of Ethnopharmacology, 79*, 331–334.

Ng, T. B., Liu, F., & Wang, Z. T. (2000). Antioxidative activity of natural products from plants. *Life Science, 66*, 709–723.

Pierre, M., Emile, M. G., & Alain, A. (1998). Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography. *Journal of Chromatography A, 800*, 171–179.

Pietta, P. G., Gardana, C., & Pietta, A. M. (2002). Analytical methods for quality control of propolis. *Fitoterapia, 73*, S7–S20.

Qi, S. D., Li, Y. Q., Deng, Y. R., Cheng, Y. Q., Chena, X. G., & Hua, Z. D. (2006). Simultaneous determination of bioactive flavone derivatives in Chinese herb extraction by capillary electrophoresis used different electrolyte systems — Borate and ionic liquids. *Journal of Chromatography A, 1109*, 300–306.

Qiao, C. F., Han, Q. B., Song, J. Z., Mo, S. F., Kong, L. D., & Kung, H. F. (2007). Chemical fingerprint and quantitative analysis of Fructus Psoraleae by high performance liquid chromatography. *Journal of Separation Science, 30*, 813–818.

Sforcin, J. M., Fernandes, A., Jr, Lopes, C. A. M., Bankova, V., & Funari, S. R. C. (2000). Seasonal effect on Brazilian propolis antibacterial activity. *Journal of Ethnopharmacology, 73*, 243–249.

State Drug Administration of China (2000). Technical request about fingerprint technology of injection of traditional medicine. *Chinese Traditional Patent Medicine, 22*(10), 671–675.

Stephen, M. B., Carol, H. C. W., Betty, Y. S., & Thomas, E. C. (2003). Identification of flavone aglycones and glycosides in soybean pods by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A, 991*, 61–68.

Strehl, E., Volpert, R., & Elstner, E. F. (1994). Biochemical activities of propolis extracts III. Inhibition of dihydrofolate reductase. *Zeitschrift fur Naturforschungc, 49*, 39–43.

Ulla, J., Pia, K., & Torben, L. (1998). Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection. *Journal of Chromatography A, 799*, 101–110.

Volpi, N., & Bergonzini, G. (2006). Analysis of flavonoids from propolis by on-line HPLC–electrospray mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 42*, 354–361.

Wang, L. X., Xiao, H. B., Liang, X. M., & Bi, K. S. (2002). Vectorial angel method for evaluating the similarity between two chromatographic fingerprints of Chinese herb. *Acta Pharmaceutical Sinica, 37*(9), 713–717.

Wang, Q. F., Cheng, Q. F., & Xu, B. (2004). The technology of extracting flavonoids from propolis. *Chinese Journal of Huaihai Institute of Technology, 13*(2), 60–62.

Wang, X., Li, F. W., Zhang, H. X., Genga, Y. L., Yuan, J. P., & Jiang, T. (2005). Preparative isolation and purification of polymethoxylated flavones from Tangerine peel using high-speed counter-current chromatography. *Journal of Chromatography A, 1090*, 188–192.

Wollenweber, E., Hausen, B. M., & Greenaway, W. (1990). Phenolic constituents and sensitizing properties of propolis, poplar balsam and balsam of Peru. *Bulletin de Groupe Polyphenols, 15*, 112–120.

Xiao, C. H., & Lu, Y. R. (1989). *Chemistry of traditional Chinese medicine*. Shanghai: Shanghai Science and Technology Press, p. 192.

Xie, P. S. (2005). *Chromatography Fingerprint of Traditional Chinese Medicine*. Beijing: People's Medical Publishing House, p. 18–104.

Yu, Z. G., Bao, B. L., Lu, B. Y., Ren, Y. P., Tie, X. W., & Zhang, Y. (2005). Determination of flavone C-glucosides in antioxidant of bamboo leaves (AOB) fortified foods by reversed-phase high-performance liquid chromatography with ultraviolet diode array detection. *Journal of Chromatography A, 1065*, 177–185.

Zhang, J. L., Cui, M., He, Y., Yu, H. L., & Guo, D. A. (2005). Chemical fingerprint and metabolic fingerprint analysis of Danshen injection by HPLC–UV and HPLC–MS methods. *Journal of Pharmaceutical and Biomedical Analysis, 36*, 1029–1035.

Zhao, J., Li, Y., Xue, X. F., & Cai, Y. (2005). The study on the parts of higher activity of the flavones contents in propolis. *Apicultural of China, 56*(3), 9–10.

Zhou, P., Zhang, Z. T., Hu, F. L., & Yu, X. Z. (2005). Primary research of HPLC fingerprints on distinguishing authentic propolis from the fake one. *Chinese Journal of Bee, 8*, 5–6.

Zhu, E. Y., Dou, Y. L., Wei, D. Z., Wang, Z. T., & Lu, Y. H. (2005). Study on HPLC fingerprint and quality control of Propolis. *China Journal of Chinese Materia Medica, 30*(18), 1423–1425.