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Quality evaluation of bee pollens by chromatographic fingerprint and simultaneous determination of its major bioactive components

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ABSTRACT

Bee pollens constitute a large number of flavonoids and thus possess great medicinal value. However different varieties of bee pollen flavonoids vary with different species and their content also differ greatly in different region. Herein, the aim of present research is to establish a method based on high performance liquid chromatography (HPLC) for quantitative analysis of flavonoids compounds and chemical fingerprint analysis of bee pollen. Five batches of rape bee pollen collected from different region of China and particularly six bee pollen species obtained in Anhui were used to establish the fingerprint. The feasibility and advantages of the used HPLC fingerprint were verified for its similarity evaluation by systematically comparing chromatograms with professional analytical software. The similarities of liquid chromatography fingerprints for five batches of rape bee pollen were more than 0.994 while six batches of different species of bee pollen were lower than 0.810. In quantitative analysis, the six compounds showed good regression ($R \geq 0.9964$) within the test ranges, and all the values for the RSD were lower than 2%. The developed HPLC fingerprint method was found simple, reliable, and it was validated for the quality control and identification of bee pollen. Additionally, simultaneous quantification of six flavonoids ingredients in the bee pollen samples was conducted to reveal the variation in their content. The results indicated that the HPLC fingerprint, as a characteristic distinguishing method combining similarity evaluation and quantification analysis, can be successfully used to assess the quality and also to identify the authenticity of bee pollen.

1. Introduction

Bee pollen is a pollen grain collected by the bees from the stamen and the gymnosperm microsporangium (Ares et al., 2018). The irregular round lump formed by the bees adding nectar, special gland secretions and saliva to the bees is rich in nutritional components and biological activity, known as “concentrated natural nutrition library” (Denisow and Denisow-Pietrzyk, 2016). The bee pollen collected by worker bees not only contains nutrients, such as proteins, fats, sugars, trace elements and various vitamins, which are usually needed by the body, but also bioactive substances such as flavonoids (Mocan et al., 2018b), carotenoids, phospholipids, and polyunsaturated fatty acids (Feas et al., 2012; Jannesar et al., 2017; Komosinska-Vassev et al., 2015). Among them, flavonoids have antibacterial, antiviral (Anusuya and Gromiha, 2019), anti-tumor, anti-oxidation (Heim et al., 2002), anti-inflammatory, analgesic (Kawai et al., 2007), anti-aging (Ferreira et al., 2018), cerebrovascular diseases (Mocan et al., 2018b), anti-radiation (Chen et al., 2016), and other pharmacological activities besides liver, heart and bones protection (Rajendran et al., 2016).

Bee pollen as apicultural product is focused for human diet because of its nutritional value. Nowadays bee pollen is used in many medical formulas and food supplements for improving health without poisonous side effects. Bee pollen stably contains flavonoids such as rutin, quercitrin, isoquercitrin, naringenin, kaempferol, and luteolin. Flavonoids are one of the major active substances in bee pollen and also serve as an important indicator of their merits (Rzepecka-Stojko et al., 2015).

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However, the content of flavonoids in bee pollen is different due to the habitats and varieties. And each bee pollen is an integrated complex with a diversity of active components, if using a single marker or a few compounds, they are not enough to complete the qualitative and quantitative analysis on the active components. Therefore, it is necessary to establish an effective method to determine the content of active components in bee pollen of different types, so as to facilitate the quality identification and evaluation of bee pollen. HPLC fingerprint method has become the most widely used method for the quality identification and qualitative evaluation of plant products due to its convenience and high efficiency (Mocan et al., 2018a; Uysal et al., 2018). The aim of this experiment was to determine the content of flavonoids in rape bee pollen of five different habitats and different varieties by HPLC fingerprint method and similarity analysis comparison, so as to evaluate the quality of bee pollen.

2. Materials and methods

2.1. Materials

Rape bee pollen were obtained from Qinghai, Qinling, Gansu, Heilongjiang and Beijing. For the comparative study, the five different species of bee pollen (i.e. leonurus pollen, Camellia flower bee pollen, Gallnut bee pollen, Rose bee pollen, and corn bee pollen) were obtained from the market in Suzhou (Anhui, China). Before the extraction, bee pollen species was identified by Prof. Jian-li Zhou, Anhui University of Traditional Chinese Medical University (He-fei, China) and sample was deposited in the herbarium of the university (Rape bee pollen for voucher number 20180801, 20180802, 20180803, 20180804, 20180805; leonurus pollen, Camellia flower bee pollen, Gallnut bee pollen, Rose bee pollen, and corn bee pollen for voucher number 20180911, 20180912, 20180913, 20180914, 20180915). The dried bee pollen grains were minced into powder through a pulverized powder machine (Xuzhong food machinery Co., Guangzhou, China) and then passed through 80 mesh sieve. The milled bee pollen powder was stored at cool and dry place at room temperature.

2.2. Reagents and chemicals

For standardization, reference compounds including rutin, isoquercitrin, quercitrin, naringenin, kaempferol, and luteolin were purchased from Qiushi bio. Sci. and Tec. Co. Ltd. (Nanjing, China). The purity of each reference compound was determined to be above 98% by HPLC analysis. HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Chemical (Waltham, CT, USA). Deionized water used for sample preparations and mobile phase was obtained by a Milli-Q Academic ultrapure water system (Millipre, Bedford, MA, USA). All other chemicals and solvents were of analytical grade.

2.3. Sample preparation

Stock solutions of six standards were prepared respectively by dissolving in methanol at concentrations of 0.251 mg/mL for rutin, 0.302 mg/mL for isoquercitrin, 0.330 mg/mL for quercitrin, 0.330 mg/mL for naringenin, 0.3410 mg/mL for kaempferol, and 0.405 mg/mL for luteolin. Then after the different standard stock solutions were mixed with a certain volume ratio and diluted to yield a series of working standard solutions at different concentrations. Stock solutions were stored in amber glass bottles at 4 °C without exposure to light over a period of at least 3 months.

Accurately weighed 2 g bee pollen powder was placed into the conical bottle, and 60 mL 75% methanol-water was added (Arruda et al., 2013; Tu et al., 2017; Zhai et al., 2018), with a solid-liquid ratio of 1:30. The bee pollen powder was extracted by ultrasonication for 60 min at temperature of 50 °C. Then the extract was cooled to room temperature, and was adjusted to the pre-extraction weight with solvent (Gao et al., 2018). The impurities in the supernatant liquid were removed successively by 0.45 μm membrane filter.

2.4. Liquid chromatography

Chromatography was performed using a Shimadzu Technologies LC series system (Kyoto, Japan) consisting of a quaternary pump, thermostated column compartment, vacuum degasser, and rheodyne injection valve with 20 μL loop, and Photo-Diode Array (PDA) (Saric et al., 2009; Zhai et al., 2014; Zhang et al., 2009). Chromatograms were recorded and evaluated by the LC Solution Lite system. The analyses were carried out on Shim Pack VP-ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm, Kyoto, Japan).

The column was maintained at 30 °C. Solvent A was acetonitrile and solvent B was 0.2% acetic acid water. The following linear gradient was applied at a flow rate of 0.8 mL/min: 0–10 min, 82%–78% B; 10–27 min, 78%–75% B; 27–32 min, 75%–70% B; 32–40 min, 70%–65% B; 40–50 min, 65%–75% B, and 50–55 min, 75%–82% B. The samples from the analytical column were monitored at a PDA absorption wavelength of 360 nm.

2.5. Method validation of quantitative analysis

2.5.1. Linearity

The calibration curves in bee pollen matrix were obtained by plotting peak areas (y) against the concentrations of the six analytes (x) in triplicate. Limit of detection (LOD) and limit of quantization (LOQ) were calculated on the basis of signal-to-noise rate of 3 (S/N = 3) and 10 (S/N = 10), respectively (Cesário et al., 2018; Pereira et al., 2018). These results we shown in Table 1.

2.5.2. Stability and repeatability

The sample solution was divided into 5 parts, while observations at 2 h, 4 h, 8 h, 16 h and 24 h were taken and over five replicates (n = 3), and the area of each peak were recorded. The results showed that the RSDs of rutin, isoquercitrin, quercetin, naringenin, kaempferol, and luteolin in the peak area were 1.42%, 1.32%, 1.81%, 1.37%, 1.95%, and 1.41%, respectively, indicating that the stability and repeatability of the solutions were appreciable up to 24 h.

2.5.3. Method validation of HPLC fingerprint analysis

The method of HPLC fingerprint analysis was validated with precision, repeatability, and stability tests. Intraday precision, repeatability, and inter-day stability of the HPLC fingerprint method were

Table 1
Linear regression data, LOQs, and LODs for the six compounds (n = 3).

Standards	Regression equation	Linger range (μg/mL)	r	LOQ (μg/mL)	LOD (μg/mL)
Rutin	$Y = 2.54 \times 10^7 X + 221, 646.57$	0.551–10.00	0.9999	0.92	2.75
Isoquercitrin	$Y = 5.71 \times 10^7 X - 164, 191.75$	0.141–6.00	0.9997	0.73	2.20
Quercetin	$Y = 4.89 \times 10^7 X - 179, 158.59$	0.183–10.00	0.9998	1.02	3.06
Naringenin	$Y = 2.99 \times 10^6 X - 12,982.90$	0.217–10.00	0.9983	0.87	2.61
Kaempferol	$Y = 1.22 \times 10^8 X - 1, 668, 129.19$	0.684–10.00	0.9989	0.21	0.63
Luteolin	$Y = 3.42 \times 10^6 X - 25, 802.426$	0.377–10.00	0.9964	0.34	1.02

Table 2
Analytical results of precision, stability, and repeatability tests of 27 characteristic common peaks in rape bee pollen samples (Sample 1) (n = 3).

Peak No.	RSD of RRT (%)			RSD of RPA (%)		
	Precision	Repeatability	Stability	Precision	Repeatability	Stability
1	0.23	0.32	0.22	0.21	0.43	0.12
2	0.28	0.13	0.27	0.08	0.12	0.21
3	0.22	0.21	0.19	0.31	0.18	0.20
4	0.09	0.11	0.13	0.21	0.17	0.13
5	0.21	0.17	0.43	0.22	0.21	0.32
6	0.31	0.24	0.21	0.19	0.16	0.20
7	0.23	0.14	0.69	0.83	0.21	0.12
8	0.33	0.21	0.14	0.13	0.17	0.25
9	0.17	0.28	0.16	0.32	0.37	0.24
10(S)	0	0	0	0	0	0
11	0.22	0.13	0.11	0.31	0.24	0.12
12	0.27	0.14	0.31	0.44	0.21	0.13
13	0.22	0.18	0.19	0.33	0.21	0.41
14	0.30	0.17	0.19	0.21	0.16	0.12
15	0.11	0.09	0.17	0.32	0.41	0.17
16	0.17	0.12	0.11	0.13	0.24	0.15
17	0.23	1.21	0.30	0.35	0.41	0.52
18	0.31	0.13	0.16	0.11	0.16	0.21
19	0.31	0.33	0.12	0.14	0.43	0.37
20	0.15	0.31	0.34	0.87	0.42	0.71
21	0.14	0.31	0.19	0.37	0.26	0.17
22	0.33	0.16	0.13	0.31	0.13	0.11
23	0.21	0.42	0.31	0.14	1.11	0.24
24	0.32	0.41	0.15	0.17	0.32	0.27
25	0.64	0.41	0.28	0.21	0.34	0.19
26	0.27	0.21	0.17	0.22	0.31	0.14
27	0.28	0.27	0.26	0.31	0.11	0.27

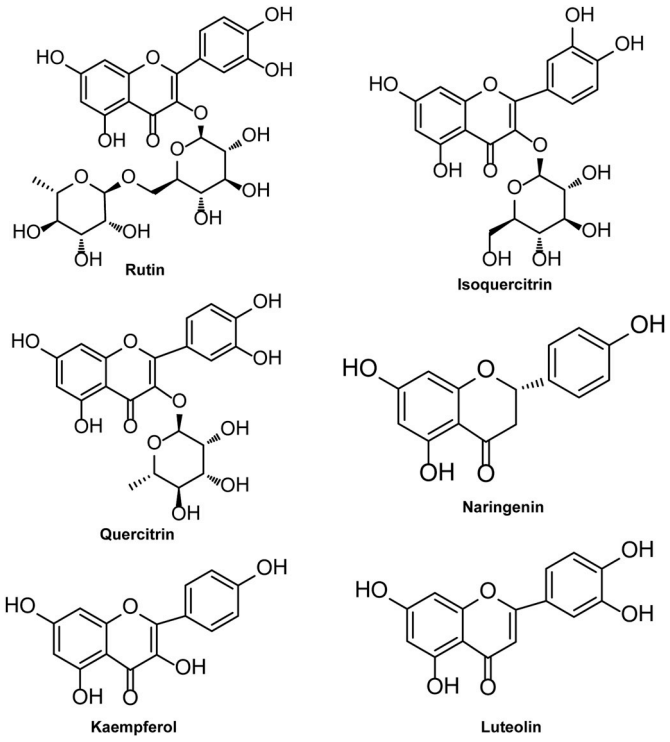

Fig. 1. The chemical structures of six selected active components of bee pollen.

determined and expressed by the relative standard deviations (RSD) value of the average relative retention times (RRT) and relative peak areas (RPA) of the 27 characteristic common peaks (Table 2). The peak 10 was selected as the reference peak to calculate the data, and the variation in the RRT and RPA of the characteristic peaks was found lower than 2.5% and 2.0% (n = 3), respectively.

3. Results and discussion

3.1. Optimization of sample pre-treatment

In order to obtain an efficient extraction of flavonoids from bee pollen, multiple related extraction conditions were optimized as described involving multiple factors including: extraction method (reflux, soak or ultrasonication), extraction solvent (ethanol and methanol), solvent concentration (50%, 75%, or 100%), solid-liquid ratio (1 : 25, 1 : 30 or 1 : 35), extraction time (30 min, 40 min, 60 min or 2 h), and extraction temperature (45 °C, 50 °C, or 60 °C). Through the orthogonal test, the results showed that the ultrasonic extraction method was superior to the other two, and the extraction effect of methanol was better than that of ethanol. The temperature showed modest effect on the extraction results, but the excessive temperature may lead to oxidation following the evaporation, resulting into misleading findings. Therefore, 50 °C was selected as an optimal extraction temperature. For perfect extraction time, it was observed that the yield was maximum within an hour, while there was not significant increase in the yield after 60 min, therefore it was taken as optimal duration for the maximum yield.

Based on these comprehensive experimental findings, the optimized scheme for the extraction was developed as 60 mL of 75% methanol to 2 g bee pollen and then ultrasound extraction for 60 min at 50 °C.

3.2. Optimization of the chromatographic conditions

The optimization of the HPLC–PDA simultaneous separation of contents in the extract of bee pollen was tested using standard solutions of six flavonoids as shown in Fig. 1. Since these six compounds obtained from bee pollen presented a broad range of polarity, gradient elution was used to achieve better analysis results. Different kinds of gradient elution of acetonitrile-water (0%, 0.2%, 0.4%, and 0.5% acetic acid) and methanol-water (0%, 0.2%, 0.4%, and 0.5% acetic acid) were investigated for bee pollen, respectively (Nascimento et al., 2018). The results showed that acetonitrile can separate all the six compounds

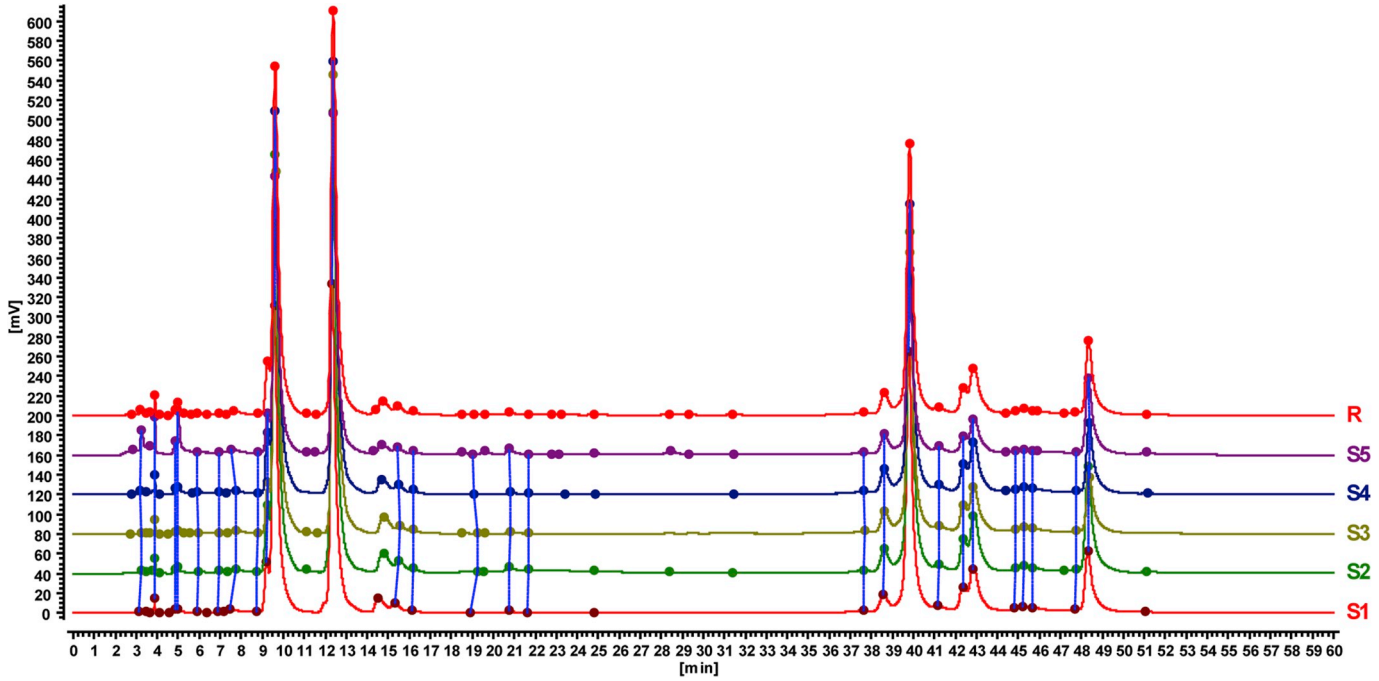

Fig. 2. HPLC chromatographic fingerprints of different origins samples. Similarities between the entire chromatographic profiles of five batches of rape bee pollen and the standard chromatographic fingerprint were calculated with Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012.130723).

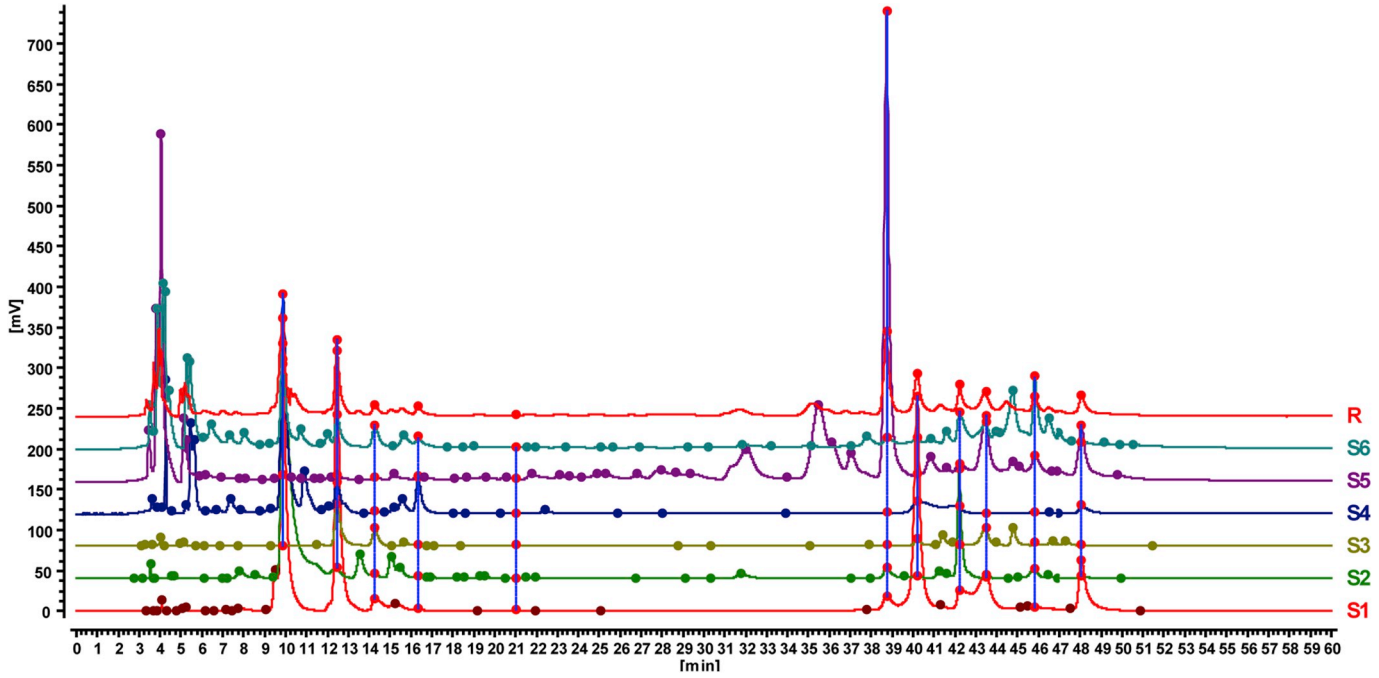

Fig. 3. HPLC chromatographic fingerprints of different species samples. Similarities between the entire chromatographic profiles of five batches of different species bee pollen and the standard chromatographic fingerprint were calculated with Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012.130723).

much efficiently as compared to methanol. To further separate the six compounds completely, we obtained the gradient elution conditions mentioned above.

3.3. Similarity analysis of HPLC fingerprints of bee pollen samples

To standardize the fingerprint, 10 samples were divided into two groups according to their origin and species followed by individual analysis. Software called Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012.130723) was used to evaluate chromatograms.

3.3.1. Similarity of different origins sample

Similarities between the entire chromatographic profiles of five batches of rape bee pollen and the standard chromatographic fingerprint were calculated with Similarity Evaluation System for

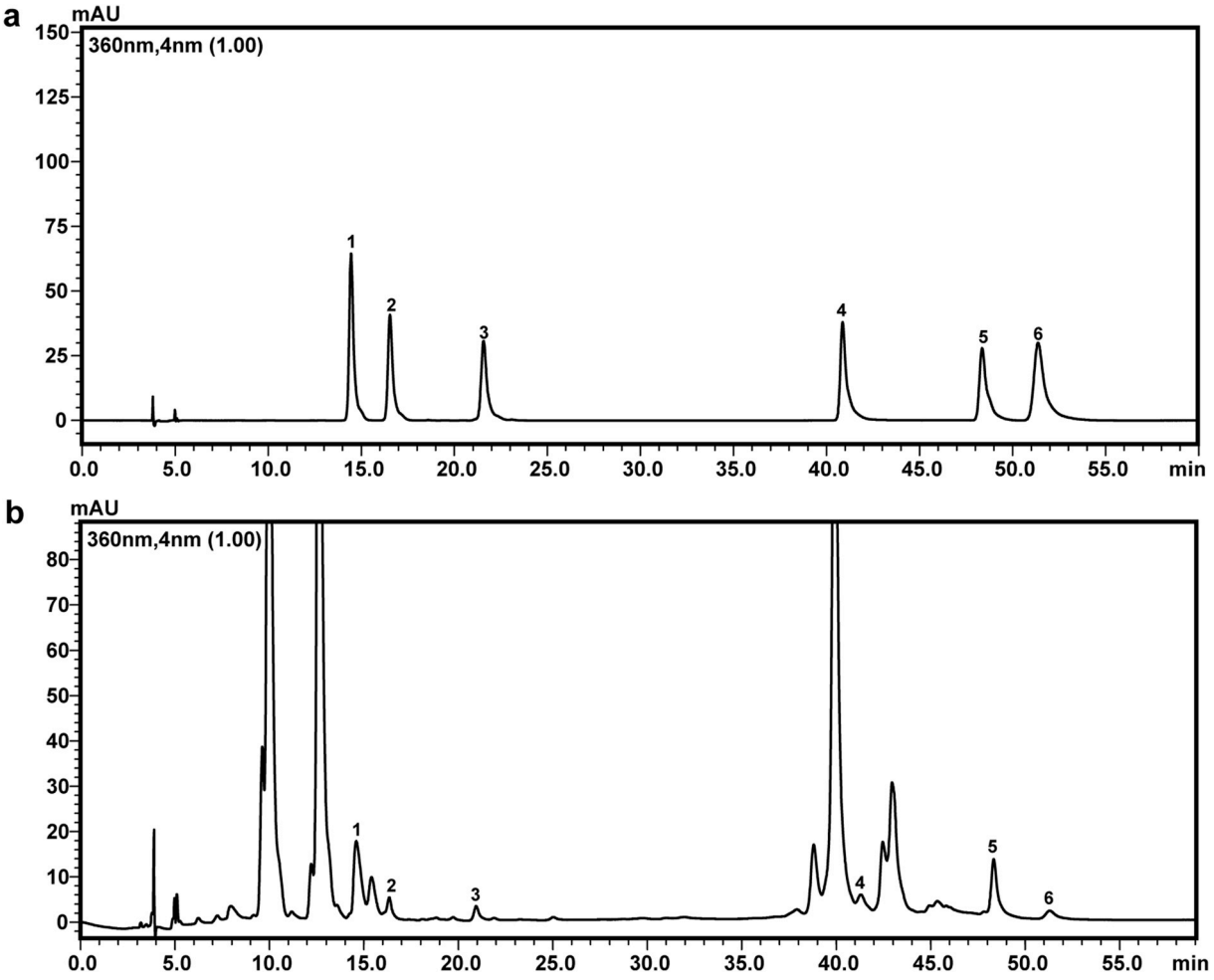

Fig. 4. HPLC chromatograms of bee pollen samples. (a) HPLC chromatogram of standard. (b) HPLC chromatogram of bee pollen sample. Key to peak identification: 1. Rutin, 2. Isoquercitrin, 3. Quercitrin, 4. Naringenin, 5.Kaempferol, and 6.Luteolin.

Table 3
Contents of the six compounds in 10 tested samples (mg/100 g) (n = 3).

Source of material	Content (mg/100 g)					
	Rutin	Isoquercitrin	Quercitrin	Naringenin	Kaempferol	Luteolin
Qinghai	1.116	0.841	0.476	2.702	3.414	0.215
Qinling	0.224	0.509	0.568	4.388	3.838	0.445
Beijing	1.476	0.478	0.607	4.271	3.666	0.173
Gansu	1.723	0.576	0.596	3.247	3.272	ND[a]
Heilongjiang	2.237	0.638	0.717	4.084	4.784	0.374
Rose	1.671	0.502	0.468	ND	1.711	ND
Gallnut	0.210	0.814	0.611	20.510	ND	ND
Leonurus	1.599	0.840	0.475	1.132	1.81	ND
corn	0.152	0.586	0.456	ND	2.023	ND
Camellia flower	0.170	0.439	0.516	5.621	ND	ND

[a] Not detected.

Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012.130723). According to the evaluation system, the similarity values of five batches of sample were 0.997, 0.998, 0.998, 1.000, and 0.994 (n = 3), respectively (Fig. 2). There were 27 characteristic peaks which were assigned as "characteristic common peaks" for the identification of the bee pollen. Five components were identified by comparing their retention time with the standard compounds. The peaks were identified as peak 11: Rutin (14.69 min), peak 13: Isoquercitrin (16.18 min), peak 15: Quercitrin (20.77 min), peak 20: Naringenin (41.20 min), and peak 27: Kaempferol (48.34 min). While the other 22 common peaks remain unidentified. These results showed that different origins rape bee pollen shared nearly the same correlation coefficients of similarities.

3.3.2. Similarity of different species sample

The relationship among samples was ascertained by comparing the similarity of the chromatographic fingerprint series of samples (Fig. 3). The correlation coefficients of all the six sample fingerprints were shown to be 0.778, 0.726, 0.451, 0.760, 0.584, 0.803, and 0.803 (n = 3). These results indicated the significant difference among the

different species of bee pollens nevertheless, they also depicted 11 common peaks. Three components among common peaks were identified by comparing their retention time with the standard compounds: peak 3: Rutin (14.69 min), peak 4: Isoquercitrin (16.18 min), and peak 5: Quercitrin (20.77 min). The remaining eight common peaks had not been identified.

3.4. Quantitative determination of flavonoids in bee pollen

As shown in Fig. 4, six peaks of active compounds from the rape bee pollen extract were obtained on chromatogram.

The contents of individual six flavonoids from bee pollen samples of different kinds are given in Table 3. It was found that the flavonoids and contents of several bee pollens differ from each other. The core focus of our study was on the rape bee pollen, in this regard we found that flavonoid content was much higher in rape bee pollen as compared to other varieties, especially produced in heilongjiang. This indicates that different production areas have great influence on the content of flavonoids in bee pollen. And other reason of difference of content may be due to different picking seasons (Negrão et al., 2014) along with geographical distribution (Avni et al., 2014; Feas et al., 2012) and plant pollen source. Earlier studies have reported that differences in nutrient content in the source pollen are the main causes of the different types and amounts of active ingredients in the bee pollen (Mandian and Tadinada, 2009; Montenegro et al., 2013).

4. Conclusions

In the present study, ultrasonic method was used to extract the flavonoids which was followed by HPLC for the determination and comparison of the content from different extracts as well as to establish the representative chromatographic fingerprint of different origin and species. For the fingerprint analysis, 27 characteristic fingerprint peaks were applied to evaluate the similarities among five different batches of rape bee pollen from different origins which showed good similarities. Six components of the 10 batches of bee pollen including different origins and species for the quantitative determination showed the difference in their content. The method developed in this study will provide an important reference to establish the quality control method for the bee pollens.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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