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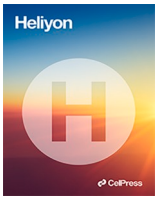

Research article

Quality control of pollen products in the market by quantitative analysis of total amino acids with liquid chromatography

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ABSTRACT

A rapid high-performance liquid chromatography (HPLC) protocol for the determination of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatization was successfully developed for assessing amino acid levels in six species of representative commercial bee pollen. Based on a poroshell column, a favorable chromatographic separation of seventeen amino acids was achieved in approximately 10 min with satisfactory resolution. The LOD and LOQ of this method were less than 0.034 μg/mL and 0.232 μg/mL, and the intra- and inter-day RSDs ranged between 0.86–5.28 % and 3.21–6.50 %, respectively. The matrix effect (ME) ranged from −8 to 3, implying that the matrix effect was not significant. Under the optimum conditions, the established method was adopted to determine amino acids in six types of bee pollens. The results showed that the total amino acid content ranged from 151.94 mg/g (*Rosa rugosa*) to 214.52 mg/g (*Leonurus artemisia*) in the six bee pollen species. Notably, proline (Pro), valine (Val), leucine (Leu), and phenylalanine (Phe) were abundant in the majority of samples. To identify the suspicious samples, principal component analysis (PCA) was performed, and each type of bee pollen was differentiated. Results showed that, in the market, the qualification rate of *RR* was 100 %, but that of *NN* was merely 62.5 %, revealing that a few of them were counterfeit. This method offers advantages such as high speed, low cost, and outstanding performance.

1. Introduction

Bee pollens, comprising pollen from various plants, refer to the male reproductive cells of flower stamens gathered by honeybees [1]. Pollen preparations are distributed worldwide for dietary purposes and as a diet supplement. Commercial bee pollen in the Chinese market is popularly consumed directly as fresh pollen granules. As a healthy apicultural product, bee pollen is considered the "only perfectly complete food", given that it contains a significant amount of essential amino acids pivotal for humans [2]. Of note, the amino acids in whole dry pollen fluctuate between 10–13 % (26.88 % protein or albuminous substances), which is equivalent to 5–7 times that of beef, milk, eggs, or cheese. Indeed, the nutritional and medicinal value of bee pollen has been established for centuries [3]. Recently, information on the chemical composition and physiological effects of bee pollens has garnered extensive interest,

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attracting worldwide attention [4,5]. As for the amino acid profile in bee pollen, current research mainly focuses on the free amino acids [6,7]. To better elucidate the nutritional value of this functional food, it is necessary to conduct an analysis of the total amino acids present.

Ascribed to fluctuations in pollen availability and price, adulteration is employed to increase the economic benefits by incorporating low-priced pollen during production and processing. Hence, there is an urgent need to pioneer a robust method to efficiently control the quality of bee pollens. Analyzing the types and contents of amino acids in pollen can offer key nutritional information to the public and manufacturers. However, the absence of specific chromophores, high polarity, and structure diversity of amino acids poses challenges in their identification and characterization [8]. Currently, there exist standardized methodologies for identifying total amino acids in foodstuffs, including the official national standard in China. Nevertheless, this conventional approach tends to be rather time-consuming and involves the utilization of ninhydrin as a derivatization reagent, which is toxic [9]. At present, techniques designed for qualitative and quantitative analysis of amino acids include ion-exchange chromatography [10], gas chromatography [11], capillary electrophoresis [12], and LC coupled with tandem mass spectrometry (MS/MS) [13,14], among which the most popular one involves a LC system coupled to a fluorescence or an ultraviolet detector [15–17]. In this procedure, the hydrophobicity of amino acids is enhanced using chromophores or fluorophores prior to separation on a reversed-phase column. Recently, several attempts have been made to optimize the effect of derivation with regents like 2,4-dinitrofluorobenzene, 9-fluorenylmethyloxycarbonyl chloride, diethylethoxymethylenemalonate, *o*-phthaldialdehyde, and so on [8,18–20]. However, these derivative methods are either toxic, slow, or produce a large number of by-products. In 1993, Cohen and Michaud first introduced 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which can react with both primary and secondary amines in seconds with strong fluorescence intensities with minimal matrix interference [21]. Afterward, efforts have been made by several research groups to enhance and adapt the AQC method to different sample matrices, such as beers, cheeses, fish, juices, and wines [22–26]. However, the method for the determination of amino acids in bee pollen remains considerably limited.

Traditional chromatographic procedures developed for a comprehensive measurement of amino acids require long running times (roughly 30 min) [27]. A sub-2 μm particle-packed column, accompanied by an ultra-high pressure LC (UPLC) system, can offer high peak efficiency, but the UPLC system and the column are not cost-effective for most research labs globally [28–30]. Recently, the use of superficially porous particles (sub-3 μm particle size) has received considerable attention. The distinctive feature of this column is that the particles consist of solid cores surrounded by a porous silica shell measuring about 0.5 μm, allowing for high peak efficiency (even at higher flow rates), low solvent consumption, and short handling time, thereby rendering this method economical and less environmentally hazardous [31,32].

In the present study, a methodology to simultaneously separate and quantify the total amino acids in several types of bee pollens, using an AQC derivation protocol followed by poroshell column-based HPLC analysis with DAD detection, was developed to address this gap. In this work, two types of column were compared, and the elution conditions were optimized to achieve a rapid separation of amino acids of interest. The optimized method was validated with matrix effects (ME), precision, linearity, sensitivity, and accuracy. Finally, the established method was applied to the quantitation of total amino acids in different bee pollens, and with the help of principal component analysis (PCA), the fake products were identified.

2. Materials and methods

2.1. Chemicals and reagents

Internal standard norleucine (Nor) and seventeen amino acid standards including phenylalanine (Phe), leucine (Leu), isoleucine (Ile), methionine (Met), valine (Val), proline (Pro), tyrosine (Tyr), cystine (Cys2), alanine (Ala), threonine (Thr), glycine (Gly), glutamic acid (Glu), serine (Ser), aspartic acid (Asp), arginine (Arg), histidine (His) and lysine (Lys) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Waters AccQ-Tag Chemistry Package consisted of AccQ-Fluor reagent kits containing borate buffer (0.4 mol/L), derivatizing reagent powder 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), reagent diluents, and AccQ-Tag Ultra Eluent A Concentrate (containing 140 mmol/L sodium acetate, 17 mmol/L triethylamine, 1 mmol/L calcium disodium EDTA, pH 5.05) was supplied by Waters Co. Ltd. (Milford, MA, USA). The acetonitrile was of HPLC grade and procured from Merck (Darmstadt, Germany). Milli-Q water made by a purification system (Millipore, Bedford, MA, USA) was used throughout the experiment.

Table 1
Information of pollen samples (n = 24).

Sample name	Date of manufacture	Specification	Origin
Leonurus artemisia (LA)	2022/10/9, 2022/10/14, 2022/10/21, 2022/11/1, 2022/11/9, 2022/11/18	230g per package	Shiyan, Hubei, China
Rosa rugosa (RR)	2022/7/24, 2022/7/28, 2022/8/1, 2022/8/6, 2022/8/25, 2022/9/15	230g per package	Shiyan, Hubei, China
Schisandra chinensis (SC)	2022/11/1, 2022/11/3, 2022/11/28, 2022/12/3, 2022/12/9, 2022/12/17	230g per package	Shiyan, Hubei, China
Nelumbo nucifera (NN)	2023/3/25, 2023/4/3, 2023/4/8, 2023/4/13, 2023/4/28, 2023/5/5	230g per package	Shiyan, Hubei, China
Camellia japonica (CJ)	2022/7/1, 2022/7/21, 2022/7/25, 2022/8/1, 2022/8/5, 2022/8/15	230g per package	Shiyan, Hubei, China
Brassica campestris (BC)	2022/11/2, 2022/11/19, 2022/12/28, 2023/1/7, 2023/1/16, 2023/1/26	230g per package	Shiyan, Hubei, China

2.2. Sample preparation

Since the outer package of pollen only indicates the production date without batch number, it is believed that the same production date means the same batch. Six batches of bee pollens (24 samples in each batch), including *Leonurus artemisia* (*LA*), *Rosa rugosa* (*RR*), *Schisandra chinensis* (*SC*), *Nelumbo nucifera* (*NN*), *Camellia japonica* (*CJ*), and *Brassica campestris* (*BC*) were acquired from Sinohive Co. Ltd., Hubei, China, authenticated by Prof. Zhifeng Zhang (School of Pharmacy, Macau University of Science and Technology) (Table 1). An additional twelve suspicious samples, whose outer packagings indicated the sample names as *NN* or *RR*, were bought from a market in mainland China and were authenticated using the method developed in this study. The preparation steps involved grinding the bee pollen samples into a fine powder and subsequent screening through a sieve with a particle size of <250 μm prior to extraction.

To obtain total amino acids, the powdered bee pollen samples (1.0000 g) were accurately weighed and mixed with 10 mL of 6 mol/L HCl containing 0.5 % phenol and hydrolysed for 24 h at 110 ± 2 °C under constant nitrogen in flame-sealed pyrex tubes. Afterward, the hydrolysate was successively filtered, and the filtrate was lyophilized and re-dissolved in 20 mL water.

2.3. Amino acid derivatization

Pre-column AQC derivatization was performed as follows: 10 μL of the filtered sample or standard solution was initially transferred to a 1.5 mL amber glass vial using a micropipette (Eppendorf AG, Hamburg, Germany). To generate a proper pH environment (8.2–10.0), 70 μL of borate buffer was introduced, and the solution was briefly vortexed. Then, 20 μL of 10 mmol/L AQC in acetonitrile was added, and the mixture was revortexed before being heated at 55 °C for 10 min in an oven. Prior to injection, samples were filtered through a 0.2 μm regenerated cellulosic membrane (Sartorius, Goettingen, Germany).

2.4. HPLC analysis

The chromatographic setup consisted of an Agilent 1260 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser unit, autosampler, quaternary pump, column heating assembly, and diode-array detector. Separation of amino acid derivatives was performed on an Agilent poroshell 120 EC-C18 column (50 × 2.1 mm, i. d., 2.7 μm) with a pre-connected 0.2 μm column inline filter. The mobile phases were composed of solvents A and B (60 % acetonitrile). Solvent A was diluted 10 times from AccQ-Tag Eluent A Concentrate. Derivatized amino acid standards or bee pollen samples were injected (2 μL injection volume) onto the column according to the following gradient: initial 8 % B; 0–4 min/8 %–10 % B; 4–8 min/10 %–32 % B; 8–12 min/32 %–45 % B; 12–15 min/45 %–100 % B. The detection wavelength was set at 254 nm, the flow rate at 0.4 mL/min, and the column temperature at 55 °C. Data acquisition was achieved using the Agilent Chemstation software package.

2.5. Statistical analysis

Statistical analysis and calculation of the mean, standard deviation, and level of significance were performed using Microsoft Excel 2007. Principal component analysis (PCA) was employed to determine the primary sources of variability within the datasets and to establish the relationship between samples (objects) and amino acids (variables). Chromatographic data were imported into the statistical software package MarkerView, version 1.2.1 (AB Sciex), to identify differences among the species of amino acids, retention time, and abundance.

3. Results and discussion

3.1. HPLC method optimization

An ideal fast liquid chromatography method aims to minimize separation time while maintaining high column efficiency. In order

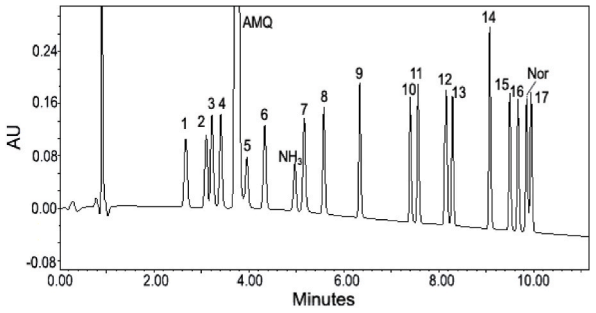

Fig. 1. HPLC chromatograms of seventeen amino acids, including 1. Asp, 2. Glu, 3. Ser, 4. His, 5. Gly, 6. Arg, 7. Thr, 8. Ala, 9. Pro, 10. Cys2, 11. Tyr, 12. Val, 13. Met, 14. Lys, 15. Ile, 16. Leu and 17. Phe.

to obtain a desirable chromatographic profile with satisfactory retention and peak shape, mobile phase, gradient elution conditions, flow rate, and column temperature were investigated.

For mobile phase, solvent A was prepared according to the suggestions of the user manual of Waters AccQ-Tag Chemistry Package, and therefore, no optimization was needed. For solvent B, acetonitrile was selected due to its better elution effect than methanol [30], and its concentration was optimized from 30 %–100 % with 10 % increment. Comprehensive considering the separation efficiency, peak shape and RT, 60 % acetonitrile was selected. With the same criterions (resolution, peak shape and RT), gradient composition and run time were studied, and the ultimate gradient program was applied as follows: initial 8 % B; 0–4 min/8 %–10 % B; 4–8 min/10 %–32 % B; 8–12 min/32 %–45 % B; 12–15 min/45 %–100 % B. Column temperature is a crucial factor as well. When the temperature was changed from 25 °C to 40 °C (5 °C increment), no obvious difference on separation efficiency and RT was observed. However, when the temperature was higher than 40 °C, both separation efficiency and RT were remarkably improved, and a best separation was achieved at 55 °C. After that, further increase in temperature resulted in an unstable baseline. Therefore, 55 °C was applied. Flow rate was investigated from 0.2 mL/min – 0.5 mL/min. Too low flow rate led to a bad separation and long RT, while too high rate caused high pressure in the column as well as a deterioration in separation. Hence, 0.4 mL/min flow rate was preferred.

Under the optimized conditions, a satisfactory separation was achieved in 10 min (Fig. 1), which was apparently more efficient than the reported methods [28–30].

3.2. Method validation

The result indicated that AQC reacts with both primary and secondary amino acids, and no significant interference was noted in the only major fluorescent by-product, 6-aminoquinoline (AMQ). The proposed method has already been validated in terms of matrix effect, precision (intra- and inter-day repeatability), linearity, sensitivity (limit of detection [LOD] and limit of quantification [LOQ]), and accuracy (recovery) in a previous study [33].

Method linearity was evaluated based on the analysis of the data with five levels of concentration by linear regression with intercepts and a 1/x weighting factor. As summarized in Table 2, the correlation coefficients (R^2) of the calibration curves were in the range of 0.9956–0.9999, which reflected acceptable linearity between the concentration (y) and the peak ratio of analytes to the internal standard (x).

Sensitivity was investigated using LOD and LOQ values, which were calculated on the basis of signal-to-noise ratio (s/n = 3 for LOD and s/n = 10 for LOQ) on the chromatography of the standard mixture. The LOD and LOQ were less than 0.034 μg/mL and 0.232 μg/mL (Table 2), signifying adequate sensitivity for conducting the determination process.

The precision of the developed method was determined in terms of intra- and inter-day variations (Table 3). Six replicates of the standard mixtures were analyzed within a day to evaluate intraday variability and were examined in triplicate for three consecutive days to assess inter-day variability. To confirm repeatability, six sample solutions were independently prepared using the previously described method. The overall intra- and inter-day variations (RSDs) of the target analytes ranged between 0.86 % ~ 5.28 % and 3.21 % ~ 6.50 %, respectively, showcasing the precision of this method.

The accuracy of the method was examined via recovery experiments. Specifically, standards corresponding to high (150 %), middle (100 %), and low (50 %) levels were spiked into 1 g of previously analyzed samples (Table S1), in which the specific addition amount of "high", "middle" and "low" was determined based on the content of each amino acid in the sample. The spiked samples were then extracted, processed, and quantified in accordance with the aforementioned methods, with the experiments performed in triplicates at each level. The average recoveries were estimated using the formula recovery (%) = [(amount found − original amount)/amount added] × 100. As depicted in Table 3, the relative recoveries of amino acids ranged from 91.8 % to 104.3 % at the three concentration levels, with RSD values lower than 4.87 %, indicating that all the analytes were well recovered. The results were consistent with the

Table 2
Parameters of calibration curves, linearity, LOD and LOQ.

Analyte	Calibration curve	R^2	Linearity range μg/mL	LOD μg/mL	LOQ μg/mL
Asp	y = 0.0504x + 0.015	0.9950	0.5–100	0.049	0.466
Glu	y = 0.0395x + 0.0149	0.9956	2–100	0.113	1.017
Ser	y = 0.0467x - 0.0029	0.9993	0.5–100	0.038	0.342
His	y = 0.0901x - 0.006	0.9987	2.5–100	0.189	1.739
Gly	y = 0.0529x + 0.0005	0.9995	1.5–100	0.077	0.689
Arg	y = 0.1945x + 0.162	0.9999	1–150	0.054	0.459
Thr	y = 0.0581x + 0.0094	0.9962	1–100	0.035	0.301
Ala	y = 0.0747x + 0.0011	0.9993	2–100	0.163	1.483
Pro	y = 0.0973x - 0.0028	0.9999	2–150	0.078	0.749
Cys2	y = 0.0801x - 0.0022	0.9999	5–100	0.233	2.144
Tyr	y = 0.0264x + 0.009	0.9977	2–200	0.134	1.430
Val	y = 0.0509x + 0.0063	0.9982	1.5–100	0.071	0.568
Met	y = 0.0271x + 0.026	0.9981	1.5–100	0.072	0.678
Lys	y = 0.0203x + 0.0195	0.9981	1–100	0.05	0.455
Ile	y = 0.0603x + 0.014	0.9976	1–200	0.034	0.232
Leu	y = 0.0598x - 0.0026	0.9990	1.5–200	0.066	0.514
Phe	y = 0.0802x - 0.0025	0.9993	1–200	0.083	0.705

Table 3
The method precision, recovery and matrix effect for poroshell based HPLC analysis of amino acids from bee pollen samples.

Analyte	Precision (RSD, %, n = 6)		Recovery (%)		Matrix effect (%)
	Intra-day	Inter-day	Mean	RSD	
Asp	3.48	3.75	94.5	2.12	−3
Glu	2.89	4.26	91.8	2.31	−5
Ser	3.82	4.36	96.3	2.08	−2
His	2.46	6.50	92.5	3.65	0
Gly	3.25	3.42	101.5	4.87	2
Arg	2.92	3.21	98.3	1.92	−6
Thr	5.28	5.32	95.6	2.88	−2
Ala	2.01	3.44	92.2	4.49	0
Pro	1.17	4.25	104.3	2.04	−3
Cys2	3.10	6.30	96.4	2.48	1
Tyr	1.84	3.22	99.2	2.22	−6
Val	0.98	4.53	100.3	1.84	−5
Met	0.86	3.87	94.8	2.43	3
Lys	2.34	5.27	93.1	1.02	−2
Ile	1.48	5.51	98.7	2.78	−4
Leu	1.93	4.38	97.4	4.42	−8
Phe	1.54	5.89	102.4	3.98	−1

accepted recovery values at certain analyte concentration levels recommended by the FDA Guidelines for the validation of Chemical Methods in the Foods Program [34].

While the separation of standard materials is relatively achievable, daily analytical tasks generally involve complex samples. The bee pollen is complicated due to its composition. Specifically, it contains not only proteins and amino acids but also vitamins, trace metals, and carbohydrates. Interferences were eliminated following sample preparation, and the established method was successfully applied to the final extracts acquired from six bee pollen samples. In this work, matrix effect (ME) was adopted to evaluate the background interference during the analysis procedure [35]. ME was defined as the suppression or enhancement of the intensity of analytes and was calculated as follows: $ME\ (\%) = (1 - Area_{\text{spiked standards in sample}}/Area_{\text{standards in pure solvent}}) \times 100\ \%$, wherein $Area_{\text{spiked standards in sample}} = Area_{\text{total amount}} - Area_{\text{original amount}}$. $ME = 0$ indicated the absence of matrix effect, whereas $ME > 0$ reflected intensity suppression, and $ME < 0$ represented enhancement. The higher the absolute ME value, the stronger the matrix interference. The matrix effect was observed in this study. As listed in Table 3, the signals of Gly, Cys2, and Met were enhanced, while the remaining signals were suppressed. The ME ranged from −8 to 3, implying that the matrix effect was not significant. As delineated in the chromatograms in Fig. 2, under the optimum conditions, the seventeen amino acids were well separated. Although the amino acid profiles substantially varied across bee pollen samples, the obtained chromatograms were of high quality, enabling amino acid profiling and quantification throughout samples, irrespective of the bee pollen species.

3.3. Pollen samples analysis

The content of amino acids in each bee pollen sample was calculated and summarized in Table 4. The values reported in this table were collected using the internal standardization method, wherein amino acid concentrations were correlated to the peak areas of amino acids and internal standards. The results demonstrated that the total amino acid contents of the six bee pollens ranged from 151.94 to 214.52 mg/g, with the highest *LA* content, followed by *SC*, *CJ*, *BC*, *NN,* and *RR*. The primary amino acids were Pro, Leu, and Phe for the *LA*, *SC*, and *CJ*, and Ser and Phe for *RR* and *BC*. In addition, *NN* was significantly different from the other five bee pollen species. For example, the content of Asp was 19.58 mg/g in *NN* and accounted for approximately 12.07 % of total amino acids, whereas it accounted for approximately 6.10–8.83 % in the other five bee pollen species. Bee pollens are typically rich in amino acids and thus can be used as nutritional substances that can supplement essential life activities and potentially prevent or cure several diseases. Therefore, further studies are warranted to investigate the relationship between amino acids and the pharmacological effects of bee pollens.

3.4. Statistical analysis

Five amino acids (Gly, Ala, Pro, Val, Leu) with better chromatographic separation and higher content were selected for analysis of variance (ANOVA), and the results are shown in Fig. 3. Among the five pollens, *LA* had the highest Gly content, whereas *RR* had the lowest. Overall, there were minimal differences in Gly content among the various pollens, as shown in Fig. 3A. Likewise, *LA* exhibited the highest Ala content, which was 66 % greater than the lowest in *RR*, as depicted in Fig. 3B. As shown in Fig. 3C, there were significant variations in Pro content among the five pollens. Notably, *SC* had the highest content, at 27.01 mg/g, which was 259 % higher than the lowest content in *NN* (7.53 mg/g). Except for *NN*, which had a Val content of 4.95 mg/g, there were minimal differences in Val content among the remaining four pollens (Fig. 3D). The Leu content was similar in *LA*, *CJ*, and *BC*, nearing 18 mg/g (Fig. 3E).

Principal component analysis (PCA) is a commonly used method to explore relationships among samples, aiming to extract a small number of latent components that summarize the measured data with minimal information loss in a reduced-dimension plot. To

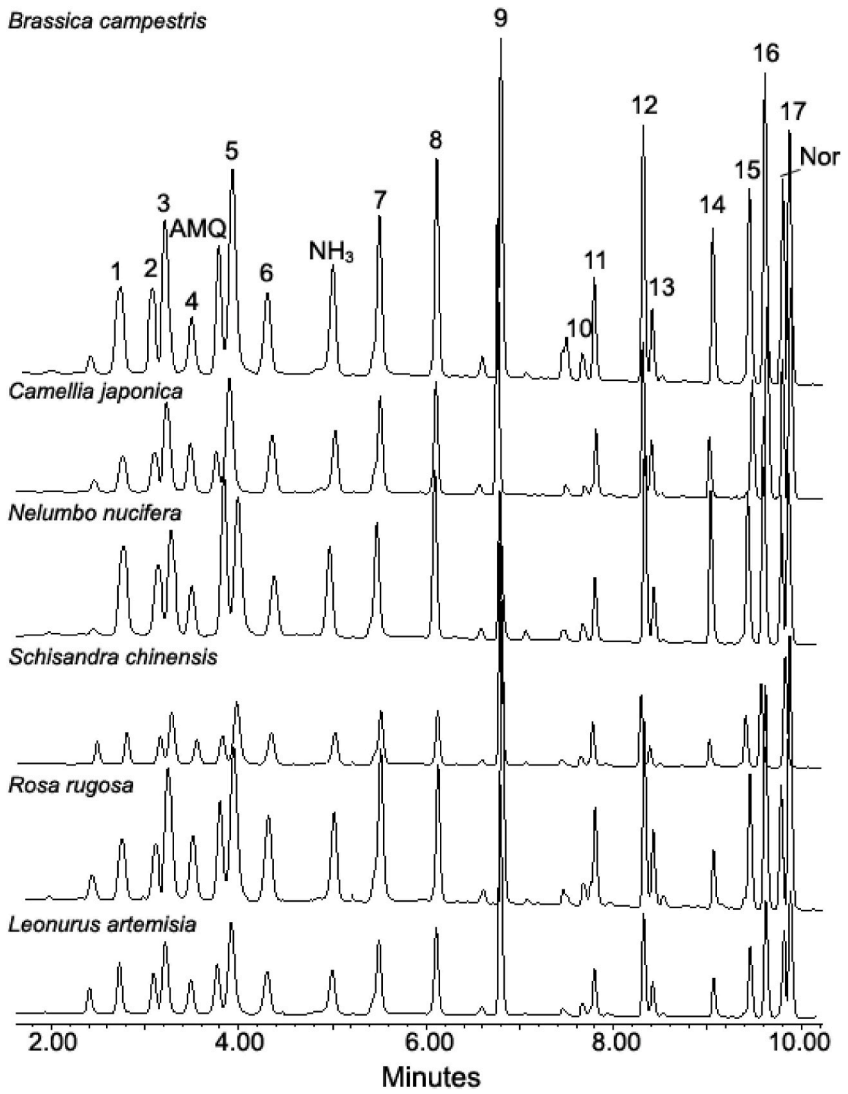

Fig. 2. HPLC chromatograms of amino acids, including 1. Asp, 2. Glu, 3. Ser, 4. His, 5. Gly, 6. Arg, 7. Thr, 8. Ala, 9. Pro, 10. Cys2, 11. Tyr, 12. Val, 13. Met, 14. Lys, 15. Ile, 16. Leu and 17. Phe, in bee pollens.

evaluate variations among bee pollen samples, PCA was performed using the contents of amino acids from HPLC profiles. The first two principal components (PC1 and PC2), which accounted for a cumulative percent (cum %) of 66.3 of the total variance in the data, were extracted for analysis. Among them, PC1 and PC2 accounted for 44.9 % and 21.4 % of the total variance, respectively. The remaining principal components were neglected, which had a marginal impact on the model. Fig. 4A illustrates the sample scatter plot, wherein each marker represents a bee pollen sample. As can be deduced, the samples were evidently clustered into several domains. *NN*, *RR*, and *BC* were significantly different from the other bee pollen species. *LA*, *CJ*, and *SC* could be partially differentiated from each other, given that some samples overlapped in the PCA plot.

In the market, *NN* and *RR* are the two most expensive bee pollens. Additionally, they are usually adulterated with some low-priced bee pollens, such as *BC*. Rece ntly, adulterated and counterfeit bee pollen has raised concerns, especially for the higher-priced *NN*, prompting an urgent need for identifying adulteration in bee pollen in the food industry. Therefore, data from the twelve suspicious samples (8 NN and 4 RR) were imported into the PCA plot in order to authenticate the bee pollens sold in the market. As shown in Fig. 4B, all four suspicious *RR* samples (blue-green points) fell within the cluster of *RR*, validating their authenticity. On the other hand, only five samples (green points) among the eight *NN* samples fell within the *NN* cluster, yielding a qualification rate of 62.5 %. The other three samples (blue points) were identified as counterfeit products, two of which were *BC*, whilst the remaining one was suspected to be *CJ*. The results indicated that this method is efficient and assists in the authentication of bee pollens in the market. Meanwhile, strict regulations concerning bee pollen should be developed. The bee pollen industry and government regulatory agencies should take proactive measures to safeguard the safety and quality of bee pollen products.

Table 4
Content of each amino acid in the six bee pollen species (mg/g).

Analyte	*Leonurus artemisia*		*Rosa rugosa*		*Schisandra chinensis*		*Nelumbo nucifera*		*Camellia japonica*		*Brassica campestris*	
	Mean value	SD	Mean value	SD	Mean value	SD	Mean value	SD	Mean value	SD	Mean value	SD
Asp	15.61	2.00	11.97	1.38	12.93	1.40	19.58	2.33	12.06	1.45	17.09	2.04
Glu	12.07	1.43	9.03	0.98	10.34	1.29	14.63	1.68	10.59	1.24	13.90	1.58
Ser	16.60	1.88	14.25	1.57	14.51	1.90	12.08	1.58	14.50	1.62	14.32	1.61
His	9.08	1.12	8.37	0.87	8.96	1.12	7.44	0.82	9.94	1.14	7.43	0.90
Gly	8.00	0.95	5.60	0.73	7.06	0.78	6.28	0.70	7.09	0.74	7.44	0.82
Arg	14.45	1.56	10.78	1.27	14.37	1.59	9.80	1.12	14.29	1.46	11.97	1.40
Thr	15.48	1.69	11.55	1.39	14.33	1.73	10.58	1.22	14.02	1.55	13.83	1.37
Ala	9.73	1.07	5.86	0.69	7.75	0.95	7.63	0.90	7.84	0.92	8.85	0.98
Pro	21.61	2.66	11.76	1.31	27.01	3.01	7.53	0.95	17.24	2.06	13.18	1.59
Cys2	3.69	0.40	1.99	0.26	3.79	0.49	1.81	0.22	2.52	0.32	3.23	0.38
Tyr	9.91	1.11	7.25	0.90	10.80	1.14	4.95	0.57	8.83	1.04	7.20	0.85
Val	16.69	2.19	12.49	1.43	15.05	1.72	14.62	1.66	17.62	1.90	16.59	1.86
Met	2.89	0.33	1.50	0.18	3.90	0.37	0.50	0.06	3.21	0.38	3.25	0.40
Lys	7.47	0.82	3.48	0.43	7.51	1.05	8.18	0.90	6.29	0.76	8.57	0.99
Ile	12.66	1.54	7.82	0.95	12.44	1.39	10.24	1.21	12.51	1.34	12.36	1.57
Leu	17.81	2.09	12.38	1.32	15.97	1.89	14.86	1.84	17.85	2.04	17.94	2.05
Phe	20.77	2.40	15.86	1.70	22.54	2.68	11.54	1.35	21.28	2.47	16.48	1.92
Total	214.52	6.73	151.94	4.41	209.26	6.56	162.25	5.29	197.68	6.18	193.63	6.09

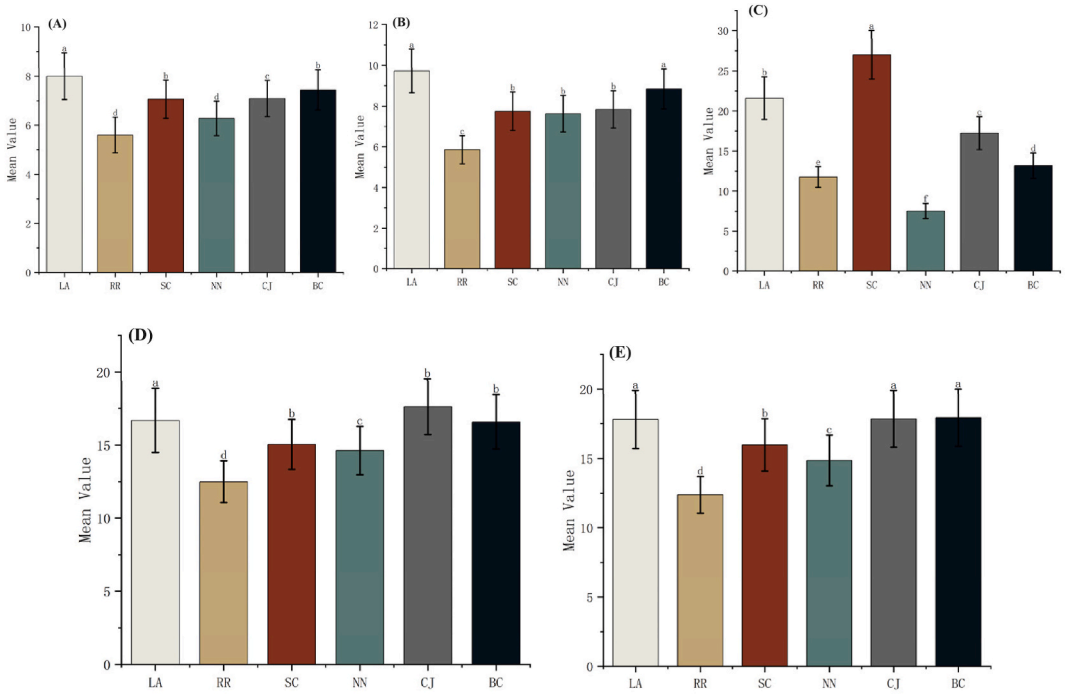

Fig. 3. Difference analysis of contents of amino acids in different pollens, including A: Gly, B: Ala, C: Pro, D: Val, and E: Leu; a ~ f: different alphabet meant significance difference ($p < 0.05$).

4. Conclusions

In summary, a fast and efficient HPLC method was developed for the determination of total amino acids in bee pollens. The application of the poroshell column lowered the analysis time to approximately 10 min on traditional HPLC equipment. The method has been fully validated and found to be precise, sensitive, and accurate. Moreover, the contents of total amino acids in six species of bee pollens, including *LA*, *RR*, *SC*, *NN*, *CJ*, and *BC*, were analyzed. After statistical analysis, the two most expensive bee pollen species, *NN* and *RR*, were clustered. The sampling survey determined that, in the market, the qualification rate of *RR* was 100 %, but that of *NN* was merely 62.5 %. This method holds considerable promise for the quality control and authentication of bee pollens and their related products.

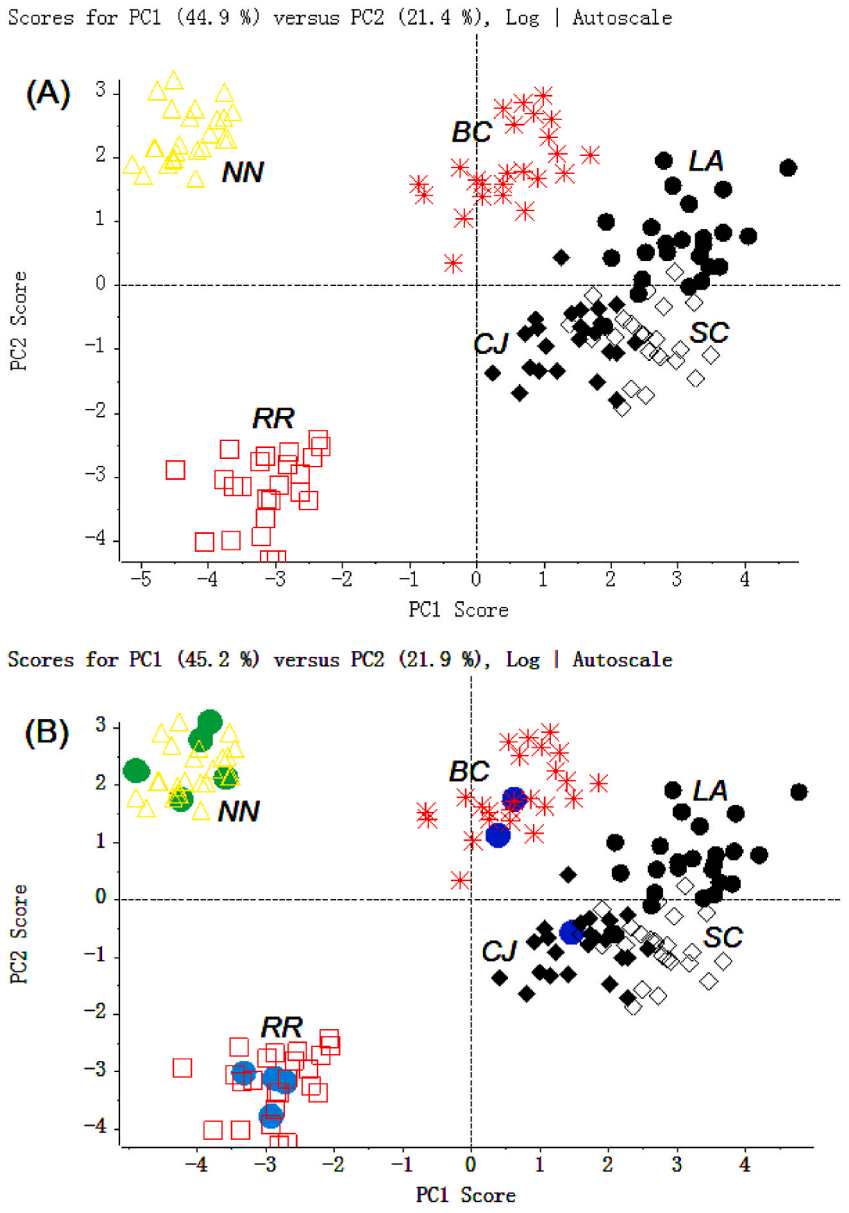

Fig. 4. (A). Principal component analysis of the seventeen amino acids in authenticated bee pollen samples. (B). Principal component analysis for fake bee pollen products detection.

Ethical approval

This article does not contain studies with human participants. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

CRediT authorship contribution statement

Linqiu Li: Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition. **Xintong Zhang:** Writing – original draft, Data curation. **Xin Wang:** Formal analysis, Data curation. **Yuan Qiu:** Formal analysis, Data curation. **Weiqiao Li:** Funding acquisition, Formal analysis. **Lianxian Guo:** Funding acquisition. **Qing Shen:** Formal analysis, Project administration. **Juanxiu Dai:** Conceptualization, Investigation, Project administration, Supervision, Validation, Visualization.

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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Abbreviation Used

AQC	*6-Aminoquinolyl-N-Hydroxysuccinimidyl-Carbamate*
AMQ	*6-aminoquinoline;*
HPLC	*High-performance liquid chromatography*
LOD	*Limit of detection*
LOQ	*Limit of quantity*
PCA	*Principal component analysis*
LA	*Leonurus artemisia*
RR	*Rosa rugosa*
SC	*Schisandra chinensis*
NN	*Nelumbo nucifera*
CJ	*Camellia japonica*
BC	*Brassica campestris*

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35833.

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