Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Quality Assessment of Pollen Typhae by High-performance Liquid Chromatography Fingerprint, Hierarchical Cluster Analysis, and Principal Component Analysis

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Submitted: 20-09-2018 Revised: 29-10-2018 Published: 06-03-2019

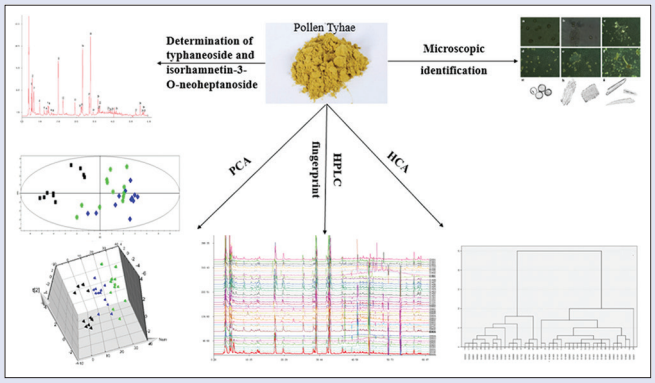
ABSTRACT

Aim: This study aims to establish the quality assessment methods of Pollen Typhae. **Materials and Methods:** High performance liquid chromatography (HPLC) fingerprint analysis, hierarchical cluster analysis (HCA), and principal component analysis (PCA) were used for quality evaluation of Pollen Typhae from different origins together with microscopic identification. Then, the quantity of 43 crude Pollen Typhae samples in the market was collected and analyzed. **Results:** In true and false test, four False Pollen Typhae samples, 13 Net Pollen Typhae (NPT) samples, and 26 Grass Pollen Typhae (GPT) samples were identified by microscopic identification. In quality test, the amounts and percentages of Qualified Pollen Typhae, Unqualified Pollen Typhae were 24 (55.81%) and 19 (44.19%), respectively with typhaneoside and isorhamnetin-3-O-neohesperidoside determined by HPLC according to China Pharmacopeia. We analyzed 43 samples from 20 regions and established their fingerprints, then selected 31 peaks as characteristic peaks and calculated their relative peak areas. To express the HPLC fingerprints quantitatively, peak 16, 18, 22, 23, and 26 were verified as typhaneoside, isorhamnetin-3-O-neoheptanoside, rutin, quercetin, and isorhamnetin. The similarity of correlation coefficients in chromatogram was 0.954 ± 0.007 and 0.922 ± 0.004 for NPT and GPT, respectively, while 0.67 ± 0.008 for 43 samples. The analysis of HCA and PCA can distinguish true or false, qualified or unqualified of Pollen Typhae. **Conclusion:** HPLC fingerprint combined with HCA and PCA provides a very efficient and comprehensive method for quality evaluation of Pollen Typhae.

Key words: Hierarchical cluster analysis, high-performance liquid chromatography fingerprint, microscopic identification, principal component analysis, Pollen Typhae

SUMMARY

The study revealed that the proposed multivariate analysis by high-performance liquid chromatography combined with a fingerprint method and analysis of principal component analysis is an efficient and comprehensive method for crude Pollen Typhae.



Abbreviations used: HCA: Hierarchical cluster analysis; PCA: Principal component analysis; FPT: False Pollen Typhae; NPT: Net Pollen Typhae; GPT: Grass Pollen Typhae; QPT: Qualified Pollen Typhae; UPT: Unqualified Pollen Typhae; RSDs: The relative standard deviations; CASE: Computer Aided Similarity Evaluation; TCM: Traditional Chinese medicine.

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DOI: 10.4103/pm.pm_469_18

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INTRODUCTION

Pollen Typhae is the dry Pollen of the genus *Typha* (*Typhaceae*) including *Typha angustifolia* L. and *Typha orientalis* Presl., which is widely distributed near the pond and shallow water in the Northern hemisphere.[1] Modern pharmacological studies have found that it has well effects in improving microcirculation, antiatherosclerosis, and antitumor.[2-4] In clinical practice, it has been widely used to prevent and treat the coronary heart disease and hyperlipidemia. Its mechanism is mainly related to reducing prothrombin time, activating coagulation factors, and increasing cyclic adenosine monophosphate levels.[5-7] Pollen Typhae contains various compounds, such as flavonoids, amino acids, steroids, fatty acids, sugars, and other types of compounds.[8] Flavonoids are main active compounds and have antioxidant, anti-inflammatory, and antigen toxic effects.[9-12] Typhaneoside and isorhamnetin-3-O-neohesperidoside are the significant components of Pollen Typhae extract. The pharmacological effects related mainly to repairing endothelial cell damage, inhibiting uterine contraction, and protecting cells from oxidative stress.[13-16] Rutin exhibits strong 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity.[17] Quercetin can clear highly reactive species such as peroxynitrite and the hydroxyl radical.[18] Isorhamnetin is known to exert beneficial effects on the prevention of obesity.[19,20] Isorhamnetin-3-O-α-L-rhamnosyl (1-2)-β-glucoside,

Cite this article as: Ma X, Zou H, Pan Y, Su J, Qiu Y, Qiu M. Quality assessment of pollen typhae by high-performance liquid chromatography fingerprint, hierarchical cluster analysis, and principal component analysis. Phcog Mag 2019;15:177-82.

quercetin, hentriacontanol-6B-sitosterol, and its palmitate play important roles in reducing lipid levels and preventing atherosclerosis.[21] Linoleic acid can strongly inhibit the binding of the Myc-Max heterodimer to E-box DNA sites.[22] Narcissin can increase the mice myocardial cell extraction of 86Rb.[23]

In previous studies, thin layer chromatography and gas chromatography-mass spectrometry are insufficient to assess the quality of Pollen Typhae. High-performance liquid chromatography (HPLC) is one of the most powerful technologies to control the quality of traditional Chinese medicine (TCM) with its simplicity, repeatability, and accuracy. HPLC fingerprint is a comprehensive and quantifiable method for identification. It is based on the research of chemical composition system of TCM, and one of the most effective analytical methods for multicomponent complex TCM.[24-30] Merely, a few researches have reported to evaluate the Pollen Typhae using HPLC. However, it is inadequate to determine only one or two markers to evaluate the overall quality of Pollen Typhae completely. Therefore, it is necessary to establish a comprehensive and systematic standard to assess the quality of Pollen Typhae. HPLC fingerprint analysis or together with hierarchical cluster analysis (HCA) and principal component analysis (PCA) for the quality assessment of Pollen Typhae have not been reported before. It may be a comprehensive evaluation method for Pollen Typhae.

In our study, we developed a simple, holistic, and reliable chromatographic HPLC fingerprint combined with PCA and HCA methods to quantitatively analyze the Pollen Typhae. This combinative method has the ability to identify false from true and unqualified from qualified and assess the quality of Pollen Typhae more comprehensively.

MATERIALS AND METHODS

Materials and reagents

We collected 43 batches of crude Pollen Typhae samples from 20 regions in China [Table 1]. Typhaneoside and isorhamnetin-3-O-neoheptanoside were provided by the Chinese Food and Drug Inspection Institute (97.0%, 93.2%, Beijing, China). Isorhamnetin, rutin, and quercetin were purchased from Chengdu Herbpurity Co., Ltd., (98.0%, 98.41%, 98.41%, Chengdu, China). HPLC grade acetonitrile was purchased from the ANPEL Scientific Instrument Co., Ltd. (Shanghai, China). Methyl alcohol was from Shanghai Lingfeng Chemical Regent Co., Ltd. (Shanghai, China), and formic acid was from Sinopharm Chemical Reagent Co., Ltd. Chloral hydrate and glycerinum were from Chemical Reagent Co., Ltd. Other chemicals were all analytical grade. Water was purified by Milli-Q System (Millipore, Merck, USA).

Apparatus

HPLC analysis was conducted with an Agilent 1200 HPLC System (Agilent Technologies, USA). The thermostatic water bath was from Shanghai Purdue HH-ZK4 Series with 220 V, 50 Hz. The analytical balance from Metller Toledo JB/T Series was used for sample weighing. OLYMPUS inverted microscope of IX51 was from Japan. Micro slides, micro coverslips of Sail Brand was made in China. The alcohol burner dissecting needle, tweezers, and lighter were dedicated to the experiment.

Microscopic identification analysis

A volume of 10 mg Pollen Typhae was dipped with dissecting needle and placed in the middle of a clean micro slide. An appropriate amount of chloral hydrate solution was added to the Pollen and mixed homogeneously. The micro slide with the sample was baked to nearly dry on the outer flame of alcohol burner. A volume of 2 mL of dilute glycerin was added and covered with a micro coverslip. Excess glycerin was absorbed by blotting paper from a side. Samples were performed in triplicate. Number the slides and record the samples information. The status of the samples was observed and recorded by the optical microscope.

Preparation of sample solution and standard solution

Each sample of crude Pollen Typhae was soaked for 1 h and extracted with eight times of methyl alcohol for 45 min at 80°C and constant pressure. All extracts were performed in triplicate and stored at −4°C for further use. The stock solution including typhaneoside and isorhamnetin-3-O-neoheptanoside, isorhamnetin, rutin, and quercetin were prepared and diluted properly. All of them were passed through 0.22 μm filters.

Table 1: Crude Pollen Typhae samples collected from different regions in China and microscopic identification results

Sample number	Region	Identification results	Sample number	Region	Identification results	Sample number	Region	Identification results
S0101	Anguo, Hebei	GPT	S0802	Guiyang, Guizhou	GPT	S1405	Shanghai	GPT
S0102	Anguo, Hebei	GPT	S0803	Shenzhen, Guangdong	NPT	S1406	Shanghai	NPT
S0201	Beijing	NPT	S0804	Zhuhai, Guangdong	GPT	S1407	Shanghai	NPT
S0301	Changsha, Hunan	GPT	S0805	Zhuhai, Guangdong	GPT	S1408	Shanghai	GPT
S0401	Zhengzhou, Henan	GPT	S0806	Zhuhai, Guangdong	GPT	S1501	Shenyang, Liaoning	NPT
S0501	Chengdu, Sichuan	NPT	S0901	Hangzhou, Zhejiang	GPT	S1601	Tianjin	FPT
S0502	Chengdu, Sichuan	GPT	S0902	Hangzhou, Zhejiang	GPT	S1602	Tianjin	GPT
S0503	Nanchong, Sichuan	GPT	S1001	Haozhou, Anhui	NPT	S1701	Weifang, Shandong	FPT
S0504	Nanchong, Sichuan	GPT	S1101	Lanzhou, Gansu	GPT	S1702	Yantai, Shandong	GPT
S0505	Nanchong, Sichuan	GPT	S1201	Nanchang, Jiangxi	NPT	S1703	Zibo, Shandong	GPT
S0601	Chongqing	NPT	S1301	Nanjing, Jiangsu	GPT	S1801	Weinan, Shanxi	NPT
S0602	Yuzhong, Chongqing	FPT	S1401	Shanghai	GPT	S1901	Wuhan, Hubei	NPT
S0701	Fuzhou, Fujian	GPT	S1402	Shanghai	NPT	S2001	Wuling, Ningxia	GPT
S0702	Xiamen, Fujian	GPT	S1403	Shanghai	NPT			
S0801	Guangzhou, Guangdong	FPT	S1404	Shanghai	GPT			

NPT stands for Pollen Typhae without staminate flower; GPT stands for Pollen Typhae with staminate flower; FPT stands for fake samples like Pollen Typhae.
GPT: Grass Pollen Typhae; NPT: Net Pollen Typhae; FPT: False Pollen Typhae

High-performance liquid chromatography conditions

The column was a TC-C18 column (4.6 mm × 250 mm, 5 μm), and the mobile phase was water-formic acid (1000:0.75) (A) and acetonitrile (B). The gradient procedure was set as follows: 5%–15% B in 0–15 min; 15%–17% B in 15–20 min; 17%–20% B in 20–30 min; 20%–77% B in 30–55 min; 77% B in 55–60 min. The flow rate was 1 mL/min, the column temperature was 30°C, the detection wavelength was 254 nm, and the injection volume was 20 μL.

Data analysis

HCA and PCA were performed with SPSS version 19.0 software (SPSS for Windows 19.0, SPSS Inc., USA) and SIMCA-P 11.5 software (Umetrics, Ume a, Sweden). The "average linkage between groups" method was applied and the cosine was selected as a measurement in HCA. The resulting three-dimensional matrix containing any specified peak index (retention time), peak intensity information (variables), and sample names (observations) was then output to the SIMCA-P software for PCA. Using the Computer-Aided Similarity Evaluation (CASE) software calculated the correlation coefficients for the entire chromatographic model and generated simulated average chromatograms and characteristic peaks.[31]

RESULTS AND DISCUSSION

Microscopic identification analysis

We processed 43 samples into microscopy specimens and analyses were performed in triplicate. Pictures of microscopy specimens were taken by the OLYMPUS inverted microscope [Figure 1]. Under the microscope, round or oval Pollen grains could be seen in Net Pollen Typhae (NPT) [Figure 1a and d]. Mesh carved lines could be observed

Table 2: Method validation for the determination of typhaneoside and isorhamnetin-3-oneohesperidin and identification of other three compounds by high-performance liquid chromatography

Peak number	Compounds	Regression equation	Test range (μg/ml)	Precision test		Repeatability test		Stability test		Recovery test	
				Peak area	RSD (%)	Peak area	RSD (%)	Peak area	RSD (%)	Average recovery rate (%)	RSD (%)
16	Typhaneoside	y=7.6074x+24.417 R^2 = 0.9997	5-500	1257.52	0.49	1450.18	1.10	1429.96	1.01	96.54	0.93
18	Isorhamnetin-3 -O-neohesperidin	y=10.957x−19.351 R^2 = 0.9993	5-500	1162.97	0.80	1990.49	1.15	2094.66	1.15	105.16	2.18
22	Rutin	-	-	153.02	0.40	77.11	1.05	101.34	0.73	-	-
23	Quercetin	-	-	175.06	0.15	103.07	1.02	69.66	0.58	-	-
26	Isorhamnetin	-	-	168.02	0.42	50.54	0.75	91.22	0.95	-	-

Each value represented in tables are means±SD (n=6). Five compounds were identified by their retention times (min): typhaneoside (29.13, peak 16), isorhamnetin-3-O-neoheptanoside (32.89, peak 18), rutin (39.93, peak 22), quercetin (42.42, Peak 23), isorhamnetin (45.53, peak 26). SD: Standard deviation; RSD: Relative standard deviations

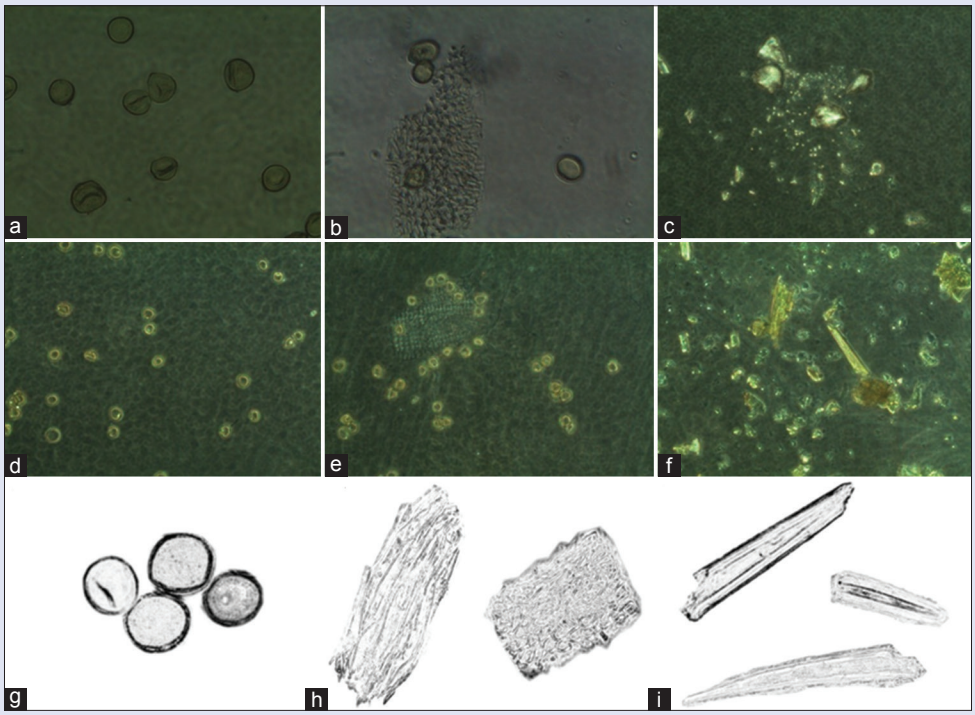

Figure 1: Microstructure of Pollen Typhae. (a and d): Microstructure of Pollen grain (10 × 40) and (10 × 20); (b and e): Microstructure of Pollen grain, calyx, and plant tissues (10 × 40) and (10 × 20); (c and f): Microstructure of false Pollen sample (10 × 40) and (10 × 20); (g) Microstructure of Pollen grain; (h) Microstructure of calyx; (i) Microstructure of duct

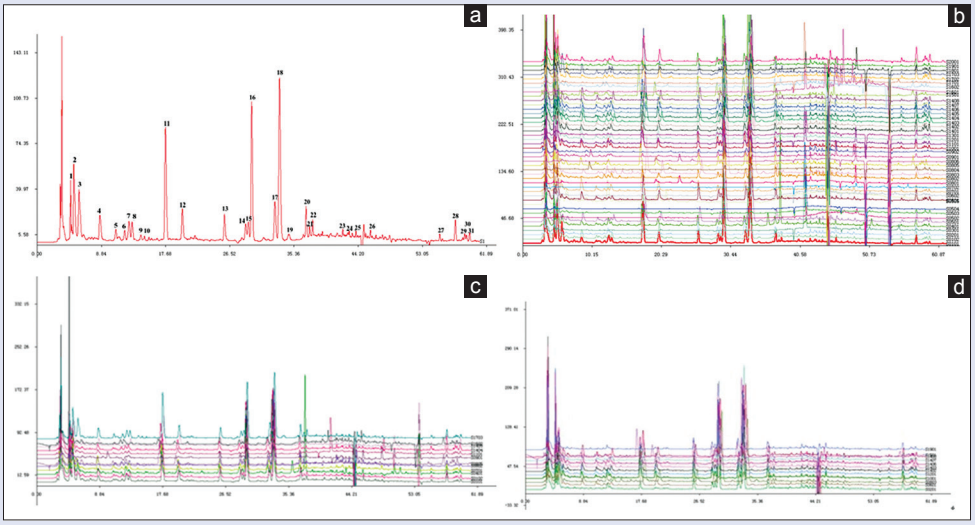

Figure 2: High performance liquid chromatography chromatograms of Pollen Typhae. (a): The number of peaks in the high performance liquid chromatography chromatograms. Typhaneoside (29.13, peak 16), Isorhamnetin-3-O-neoheptanoside (32.89, peak 18), Rutin (38.93, peak 22), Quercetin (42.42, Peak 23), Isorhamnetin (45.53, peak 26); (b) high performance liquid chromatography chromatograms of 43 Pollen Typhae from 20 regions of China; (c) high performance liquid chromatography chromatograms of 13 Net Pollen Typhae samples; (d) high performance liquid chromatography chromatograms of 26 Grass Pollen Typhae samples

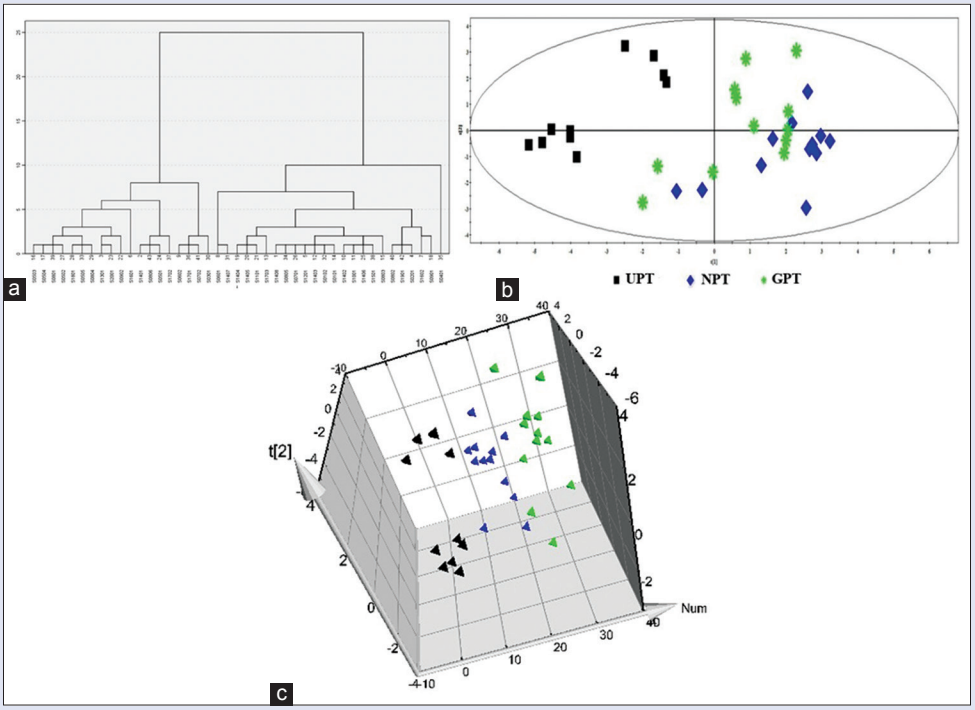

Figure 3: Results of multi-dimensional analysis of 43 Pollen Typhae samples. (a): Dendrograms of hierarchical cluster analysis of 43 samples; (b) principal component analysis of Unqualified Pollen Typhae, Grass Pollen Typhae and Net Pollen Typhae; (c) three dimensional-principal component analysis figure of Unqualified Pollen Typhae, Grass Pollen Typhae, and Net Pollen Typhae

on the surface of the Pollen grain. The peripheral contour line of Pollen grain was smooth with wavy or convex gear [Figure 1g]. A small amount of plant tissue such as pollen, calyx, and duct could be seen in some samples called Grass Pollen Typhae (GPT) [Figure 1b and e]. Moreover, a mass of plant tissue and unknown crystal could be observed in the False Pollen Typhae (FPT) [Figure 1c and f]. There were a total of 26 GPT samples, 13 NPT samples, and four FPT samples in 43 samples [Table 1]. The above results showed that the GPT, NPT, and FPT were existed in the market. The FPT accounted for a proportion of 9.3%. Therefore, the evaluation of the quality of Pollen Typhae is necessary. The microscopic identification analysis is a good way to tell false from true of Pollen Typhae and tell NTP from GTP in true samples.

Method validation

As shown in Table 2, the relative standard deviations (RSDs) of precision test ranged from 0.15%–0.80% ($n = 6$). The RSD of repeatability test varied from 0.75% to 1.15% ($n = 6$). The stability of the solutions was determined at 0, 2, 4, 8, 12, 24, and 48 h, and the RSD of the stability teat ranged from 0.58% to 1.15%. The average recovery rate of the typhaneoside and isorhamnetin-3-O-neoheptanoside were 96.54% ± 0.03% and 105.16% ± 0.02%, respectively. Moreover, the RSD varied from 0.93% to 2.18% ($n = 6$). The above results revealed the repeatability and accuracy of the analysis conditions.

Determination of typhaneoside and isorhamnetin-3-O-neoheptanoside

The determination of typhaneoside and isorhamnetin-3-O-neoheptanoside in samples from different regions were analyzed by HPLC according to China Pharmacopoeia (2015 edition)[31] [Table 3]. By comparing the retention time with standard substances, peaks 16 and 18 were identified as typhaneoside and isorhamnetin-3-O-neoheptanoside, respectively [Figure 2]. The contents of typhaneoside and isorhamnetin-3-O-neoheptanoside varied from 0% to 0.58% (g/g) and from 0% to 0.55% (g/g), respectively. The samples, whose total percentage of typhaneoside and isorhamnetin-3-O-neoheptanoside did not reach 0.5%, were called Unqualified Pollen Typhae (UPT). Moreover, the rest of the samples were called Qualified Pollen Typhae (QPT). In this study, the quantity and the proportions of UPT and QPT samples were 19 (44.19%) and 24 (55.81%), respectively. UPT accounted for nearly half of the proportion.

High performance liquid chromatography fingerprint analysis

To evaluate the quality of Pollen Typhae comprehensively, we analyzed 43 samples from different regions and constructed a standard HPLC fingerprint [Figure 2a and b]. We selected and marked 31 common peaks as characteristic peaks. Compared retention time and ultraviolet spectrum with standard references, peak 16, 18, 22, 23, and 26 were in conformity with typhaneoside, isorhamnetin-3-O-neoheptanoside, rutin, quercetin, and isorhamnetin. Peak 18 (isorhamnetin-3-O-neoheptanoside) was selected as the reference peak because its proportion of areas accounted of 10%. In addition, the similarity of the chromatogram was evaluated by CASE software.

The correlation coefficients of similarity in chromatogram were 0.954 ± 0.007, 0.922 ± 0.004 for NPT and GPT, respectively [Figure 2c and d], while 0.67 ± 0.008 of 43 samples. The results above showed that the quality of the samples in the market had a certain difference due to the wide distributions and different varieties.

Hierarchical cluster analysis of the samples

In the light of the chromatograms of all samples, the natural cluster was found by HCA to assess the differences of Pollen Typhae from 20 regions. When the clustering coefficient was 5, 43 samples were split into eight groups [Figure 3a]. Compared above, the results of these eight groups were consistent with that of microscopic identification and measurement. In summary, HCA can differentiate the superior or inferior of the samples.

Principal component analysis of the samples

PCA was considered as a data reduction technique to produce a visual scatterplot for the qualitative assessment of resemblances and differences among the samples. To further classify the true samples of Pollen Typhae without FPT, PCA was performed [Figure 3b and c]. In the figure,

Table 3: Determination of typhaneoside and isorhamnetin-3-O-neoheptanoside in 43 Pollen Typhae samples

Sample number	Typhaneoside (%)	Isorhamnetin-3-O -neoheptanoside (%)	Total content (%)	Sample number	Typhaneoside (%)	Isorhamnetin-3-O -neoheptanoside (%)	Total content (%)	Sample number	Typhaneoside (%)	Isorhamnetin-3-O -neoheptanoside (%)	Total content (%)
S0101	0.20	0.31	0.51	S0802	0.27	0.29	0.56	S1405	0.34	0.33	0.67
S0102	0.31	0.23	0.54	S0803	0.22	0.28	0.50	S1406	0.26	0.31	0.57
S0201	0.58	0.52	1.10	S0804	0.14	0.15	0.29	S1407	0.35	0.55	0.90
S0301	0.00	0.00	0.00	S0805	0.27	0.24	0.51	S1408	0.37	0.39	0.76
S0401	0.22	0.33	0.55	S0806	0.23	0.20	0.43	S1501	0.18	0.60	0.78
S0501	0.19	0.24	0.43	S0901	0.56	0.55	1.11	S1601	0.00	0.00	0.00
S0502	0.00	0.00	0.00	S0902	0.23	0.24	0.47	S1602	0.26	0.24	0.5
S0503	0.00	0.00	0.00	S1001	0.24	0.29	0.53	S1701	0.00	0.00	0.00
S0504	0.00	0.00	0.00	S1101	0.34	0.20	0.54	S1702	0.06	0.10	0.16
S0505	0.04	0.04	0.08	S1201	0.32	0.32	0.64	S1703	0.42	0.44	0.86
S0601	0.39	0.13	0.52	S1301	0.16	0.16	0.32	S1801	0.06	0.06	0.12
S0602	0.00	0.00	0.00	S1401	0.18	0.19	0.37	S1901	0.35	0.2	0.55
S0701	0.37	0.15	0.52	S1402	0.31	0.23	0.54	S2001	0.08	0.11	0.19
S0702	0.00	0.00	0.00	S1403	0.45	0.48	0.93				
S0801	0.00	0.00	0.00	S1404	0.38	0.41	0.79				

Each value represented in tables are mean±SD (n=3). SD: Standard deviation

the black dots stood for UPT samples, the green dots (GPT), and the blue dots (NPT) were on behalf of QPT samples. They can be found to be clearly separated from each other. The GPT and NPT could be basically separated with R2Ycum = 0.559 represented interpretation and Q2Y = 0.366 stood for forecast [Figure 3b]. In the three-dimensional-PCA figure, the three groups could be completely separated [Figure 3c], which might suggest of some differences between UPT and QPT. And QPT can be divided into GPT and NPT.

CONCLUSION

In this article, 43 samples of Pollen Typhae from 20 regions were analyzed by HPLC fingerprint analysis, HCA and PCA together with optical microscope. In previous studies, traditional microscopic identification can tell false from true, tell GPT from NPT, but it cannot confirm whether the crude Pollen Typhae is qualified or not. Two compounds determination only can evaluation its quality partly. Either of them cannot evaluate the quality of Pollen Typhae comprehensively. Because of the high similarity of chromatograms among samples, it is necessary to introduce novel methods to solve this problem. The HPLC fingerprint is a good choice to evaluate the quality of the samples comprehensively. The HCA and PCA can analyze the complex data column of HPLC fingerprint. We managed to divide 43 samples into different groups including UPT group and QPT group. Moreover, QPT group was divided into two groups of GPT and NPT.

In conclusion, HPLC coupled with multivariate analysis was developed to evaluate the quality of Pollen Typhae comprehensively. And, HPLC-HCA or HPLC-PCA can be used to classify the samples of Pollen Typhae. The methods have been successfully applied in the evaluation of Pollen Typhae samples in the market. The study revealed that the proposed multivariate analysis by HPLC combined with a fingerprint method, and analysis of PCA is an efficient and comprehensive tool for crude Pollen Typhae.

Acknowledgement

It was supported by the Shanghai Science and Technology Commission Project 14401900100, 1540190170, and 17401900900.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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