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Comprehensive analysis of Polygoni Multiflori Radix of different geographical origins using ultra-high-performance liquid chromatography fingerprints and multivariate chemometric methods

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ABSTRACT

Polygoni Multiflori Radix (PMR) is increasingly being used not just as a traditional herbal medicine but also as a popular functional food. In this study, multivariate chemometric methods and mass spectrometry were combined to analyze the ultra-high-performance liquid chromatograph (UPLC) fingerprints of PMR from six different geographical origins. A chemometric strategy based on multivariate curve resolution–alternating least squares (MCR–ALS) and three classification methods is proposed to analyze the UPLC fingerprints obtained. Common chromatographic problems, including the background contribution, baseline contribution, and peak overlap, were handled by the established MCR–ALS model. A total of 22 components were resolved. Moreover, relative species concentrations were obtained from the MCR–ALS model, which was used for multivariate classification analysis. Principal component analysis (PCA) and Ward's method have been applied to classify 72 PMR samples from six different geographical regions. The PCA score plot showed that the PMR samples fell into four clusters, which related to the geographical location and climate of the source areas. The results were then corroborated by Ward's method. In addition, according to the variance-weighted distance between cluster centers obtained from Ward's method, five components were identified as the most significant variables (chemical markers) for cluster discrimination. A counter-propagation artificial neural network has been applied to confirm and predict the effects of chemical markers on different samples. Finally, the five chemical markers were identified by UPLC–quadrupole time-of-flight mass spectrometer. Components 3, 12, 16, 18, and 19 were identified as 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside, emodin-8-O-β-D-glucopyranoside, emodin-8-O-(6′-O-acetyl)-β-D-glucopyranoside, emodin, and physcion, respectively. In

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conclusion, the proposed method can be applied for the comprehensive analysis of natural samples.

1. Introduction

Polygoni Multiflori Radix (PMR) is the root of *Polygonum multiflorum* Thunb., which has long been used in the treatment of hyperlipidemia in China. Nowadays, PMR is increasingly being used not only as a traditional herbal medicine but also as a popular functional food in Asian countries [1–3]. Stilbenes, anthraquinone, polysaccharides, tannins, and flavonoid glycosides are the main components of PMR [4–6], possessing multivariate pharmacological activities, such as laxative activity, lipid regulation effect, antioxidant activities, antiaging properties, immune-enhancing properties, hepatoprotective activity, and inducing hair growth [6–10]. In addition, novel functional polysaccharides have been found from PMR water-extracted residue, which have immunomodulatory activity and can be applied as potential immunomodulators [11].

The multivariate pharmacological activities of PMR is attributed to the chemical components contained, however, many factors influence the contents of components in natural herbs, including geographical region, species variation, harvesting time, processing, and so on, thus making quality assurance a challenging undertaking in research [12–14]. It is essential to establish sensitive and accurate methods to control the quality of natural samples. A so-called fingerprint can reflect the characteristic profile of the samples, of which the chromatographic fingerprint is the most widely used tool to analyze natural samples [15]. However, there are common problems in chromatographic fingerprinting, including low signal-to-noise ratio (S/N), peak overlap, non-negligible background contribution, and so on. In particular, peak overlap greatly reduces the accuracy of quantitative results. Application of a chemometric strategy can successfully handle the common problems in chromatographic fingerprinting and can yield useful information even when complete separation is not achieved [16]. Multivariate curve resolution (MCR) is a widely used and powerful methodology to analyze and model multiway data in different fields [17]. Tauler proposed MCR by alternating least squares (MCR–ALS), which is a flexible MCR algorithm [18,19]. In addition, MCR–ALS is constantly being improved and updated, making it increasingly applicable in different fields [20,21].

Many studies of PMR have been focused on its chemical and pharmacological properties. The multivariate chemical markers can provide a comprehensive evaluation of functional food quality [22]. However, few studies have applied multivariate chemometric methods to comprehensively analyze the chromatographic fingerprints of PMR and obtained chemical markers from different geographical origins. In this study, a chemometric strategy has been developed, including MCR–ALS and multivariate classification methods, to comprehensively analyze the ultra-high-performance liquid chromatograph (UPLC) fingerprints of PMR from different geographical origins. MCR–ALS GUI 2.0 has been applied to develop the MCR–ALS model and to obtain useful information, such as pure elution profiles, spectral profiles, relative species concentration, and so on. Multivariate classification methods have been applied to analyze samples from six different regions. Principal component analysis (PCA) and Ward's method were first used to classify 72 PMR samples [23–25]. Most important components (chemical markers) among all compounds were obtained from Ward's method. A counter-propagation artificial neural network (CP-ANN) was applied to predict the effects of these chemical markers on different samples [26–28]. Finally, the chemical markers were identified using UPLC–quadrupole time-of-flight mass spectrometer (UPLC–Q-TOF-MS) and retention times were obtained from standards. The proposed strategy provides a more comprehensive way to evaluate quality of functional foods from different geographical regions.

2. Methods

2.1. Materials and reagents

PMR samples were collected from six different provinces (Anhui, Guangdong, Guangxi, Guizhou, Sichuan, and Henan) of China. HPLC-grade methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade formic acid was obtained from Tianjin Kemiou Chemical Reagent Co., Ltd. Water used as a chromatographic mobile phase was purified using a Milli-Q system (Millipore, Bedford, MA, USA). 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside, emodin, and physcion were purchased from Chinese Authenticating Institute of Material and Biological Products (Beijing, China). Emodin-8-O-β-D-glucopyranoside was obtained from Sichuan Weikeqi Biological Technology Co., Ltd (Sichuan, China).

2.2. Sample preparation

Samples of PMR from different geographical regions were collected, finely powdered, and passed through a 24-mesh sieve. Then, 100 mg of each powder sample was dissolved in methanol (5 mL) and sonicated for 15 minutes. All samples were examined in triplicate and were coded according to Table 1.

2.3. UPLC and UPLC–Q-TOF-MS analysis

A Waters ACQUITY UPLC system equipped with a Xevo G2-XS Q-TOF mass spectrometer (Waters, Milford, MA, USA) was

Table 1 – Description of Polygoni Multiflori Radix samples.

Samples	Sample code	Geographical origin	Origin code
1–15	AH11-AH12-AH13-AH21-AH22-AH23 AH31-AH32-AH33-AH41-AH42-AH43 AH51-AH52-AH53	Anhui, China	AH
16–24	GD11-GD12-GD13-GD21-GD22-GD23 GD31-GD32-GD33	Guangdong, China	GD
25–33	GX11-GX12-GX13-GX21-GX22-GX23 GX31-GX32-GX33	Guangxi, China	GX
34–45	GZ11-GZ12-GZ13-GZ21-GZ22-GZ23 GZ31-GZ32-GZ33-GZ41-GZ42-GZ43	Guizhou, China	GZ
46–63	SC11-SC12-SC13-SC21-SC22-SC23 SC31-SC32-SC33-SC41-SC42-SC43 SC51-SC52-SC53-SC61-SC62-SC63	Sichuan, China	SC
64–72	HN11-HN12-HN13-HN21-HN22-HN23 HN31-HN32-HN33	Henan, China	HN

used to identify chemical markers. The chromatographic separation was performed on an ACQUITY UPLC BEH shield RP_{18} column (100 mm × 2.1 mm, 1.7 μm particle size; Waters), operated at 45°C. The mobile phase was composed of methanol (Solvent A) and water containing 0.1% formic acid (Solvent B). A gradient elution program was employed as follows: 0–5 minutes (10–35%, A), 5–20 minutes (35–90%, A), 20–21 minutes (90–100%, A) with a mobile phase flow rate of 0.3 mL/min. The UV spectra were recorded from 210 nm to 400 nm at 0.615 nm increments, and sampling rate of the detector was set as 20 points/s. The injection volume was 3 μL. The MS analysis was performed using an electrospray ionization source in negative-ion mode, and the range of full-scan mass was 100–1000 Da. Source temperature and desolvation temperature were 100°C and 400°C, respectively. The desolvation gas flow was set at 600 L/h. The voltage of capillary and cone were set at 3 kV and 40 V, respectively. Leucine–enkephalin (*m/z* 556.2771)/(*m/z* 554.2615) was chosen as a reference mass. All solutions were further filtered through a 0.22-μm nylon membrane prior to injection to the UPLC and UPLC–Q-TOF-MS systems.

2.4. Chemometric analysis

MCR is a powerful methodology, and has been widely applied in data analysis in many different fields. It involves decomposing the data matrix into a bilinear model to extract relevant information on the pure components from a mixed system [17,20]. MCR–ALS is an iterative method based on MCR [29], which allows for simultaneous analysis of independent and different runs by the same instrument [20], and its equation can be written as follows:

$$\begin{bmatrix} D_1 \\ \vdots \\ D_n \end{bmatrix} = \begin{bmatrix} C_1 \\ \vdots \\ C_n \end{bmatrix} S^T + \begin{bmatrix} E_1 \\ \vdots \\ E_n \end{bmatrix} \quad (1)$$

where C matrix, S^T matrix, and E represent row mode profiles, column mode profiles, and the matrix of residual, respectively. A column-wise augmented matrix is simultaneously generated in this data arrangement, where concentration profiles may be different, but the resolved pure spectra are the same.

The column-wise augmented matrix of each segment was analyzed utilizing MCR–ALS GUI 2.0 under optimum constraints, which is an updated version that includes not only recently published advances, but also an increased number of options and some new functions [20]. First, data for PMR samples were obtained in American Standard Code for Information Interchange (ASCII) format from the Waters ACQUITY UPLC, and then these data were exported into the MATLAB R2014a environment. Each chromatographic fingerprint data set was segmented by utilizing a local rank analysis method of evolving factor analysis (EFA), which can provide information about different regions, such as overlapped, zero-component, and selective regions. The singular value decomposition algorithm was chosen to determine the number of components; initial estimates were obtained by the EFA method or by a purest variable detection method. Non-negativity using the final algorithm was selected for constraints of row- and column-mode profiles. ALS optimization was performed, and the output information that was obtained included spectra, relative species concentration, fitting error (lack of fit), percent of variance explained at the optimum, and so on, based on which the best MCR–ALS model could be obtained for each chromatographic segment.

The relative species concentrations obtained from the output information were selected to build a new matrix for analysis, which contained all the resolved components of all the PMR samples. Multivariate chemometric classification methods, including PCA and Ward's method, were applied to classify the new matrix data, show the similarities and differences, and identify the marker components. A CP-ANN was used to confirm the results of PCA and Ward's method, and to study the effect of five chemical markers in the discrimination of different samples. Finally, the marker components were identified by a combination of UPLC–Q-TOF-MS, MCR–ALS, and standards.

2.5. Software requirements

All the chemometric methods were executed and coded in MATLAB R2014a (MathWorks, Natick, MA, USA). MCR–ALS GUI 2.0 (the GUI-updated version for the MCR–ALS algorithm, developed by Joaquim Jaumot et al., Barcelona, Spain) [20], Kohonen and CP-ANN toolbox version 3.6 (Milano Chemometrics and QSAR Research Group, University of Milano Bicocca, Milano, Italy) [26,27], and PLS-Toolbox version 7.9 (Eigenvector Research, Wenatchee, WA, USA) were used for analysis as implemented under MATLAB soft.

3. Results and discussion

3.1. Validation of the methodology

Precision and repeatability were determined to evaluate the UPLC methodological stability. For each chromatogram obtained, the retention time and areas of six selected peaks were retrieved, which span the whole region with good separation. The method precision was obtained by assaying the same PMR sample six times during a single day; the relative standard deviation values for retention time did not exceed 0.11%, and the highest value for areas was 1.42%. Six independent portions from a sample were prepared and analyzed in parallel for the method repeatability; the relative standard deviation values for retention time and areas were less than 0.12% and 1.85%, respectively. All the results showed good stability of the UPLC method used.

3.2. MCR–ALS modeling

The raw UPLC fingerprints at 260 nm of 24 PMR samples from six different provinces of China are shown in Figure 1. As can be seen, it does not have significant retention time shifts, but presents the phenomena of peak overlap present in several periods, especially at 12.85–13.3 minutes. Moreover, low S/N was also apparent in the UPLC–photodiode array detection fingerprints of PMR samples. In this work, the MCR–ALS model established by MCR–ALS GUI 2.0 was proposed to deal with these chromatographic problems, and pure elution profiles and spectral profiles of components of PMR in different samples were obtained by this method. To speed up the analysis process, the UPLC fingerprints were segmented into 15 common chromatographic regions (i.e., segments a–o) based on the rank map achieved by the EFA method. Column-wise augmented matrices for each segment for 72 samples were established to provide bilinear data for analysis by MCR–ALS GUI 2.0. The singular value decomposition algorithm was first chosen to select the number of components; initial estimates were obtained by a purest variable detection method, and the purest spectra were chosen with a noise level of 5%. Non-negativity using the final algorithm was selected for constraints of row- and column-mode profiles. Taking segment k for example, Figure 2 shows the analysis results obtained from MCR–ALS GUI 2.0. Resolved augmented elution profiles for 72 PMR samples are depicted in Figure 2A. It can clearly be seen that the background, baseline contribution, and overlap peak were resolved by MCR–ALS GUI 2.0. As can also be seen from Figure 2A, there were various baselines for the chromatographic segments detected and modeled by the method. There was obvious overlap in segment k (i.e., at retention times of 750–820 seconds), but the overlapping peak was successfully resolved. Figures 2B and 2C show the pure elution profiles for sample AH11 and resolved spectral profiles, respectively. The MCR–ALS model was clearly capable of adequately resolving the chromatographic problems, and despite overlapping peaks, the spectral profiles of each chemical component could be obtained. Each chromatographic segment was resolved by MCR–ALS GUI 2.0 in a common way. The output information obtained included pure elution profiles, spectral profiles, areas of species concentration, and relative species concentrations. The lack-of-fit values in all cases were below 8%, and the percent of variance explained exceeded 99.5%, which was acceptable based on the complexity and noise level of the data.

3.3. Classification of chromatographic fingerprints

The relative species concentrations of resolved chemical components in the different samples were obtained from the output information, which were important elements to create a new data matrix (with the dimensions 72 samples × 22 variables). The data matrix was analyzed by multivariate

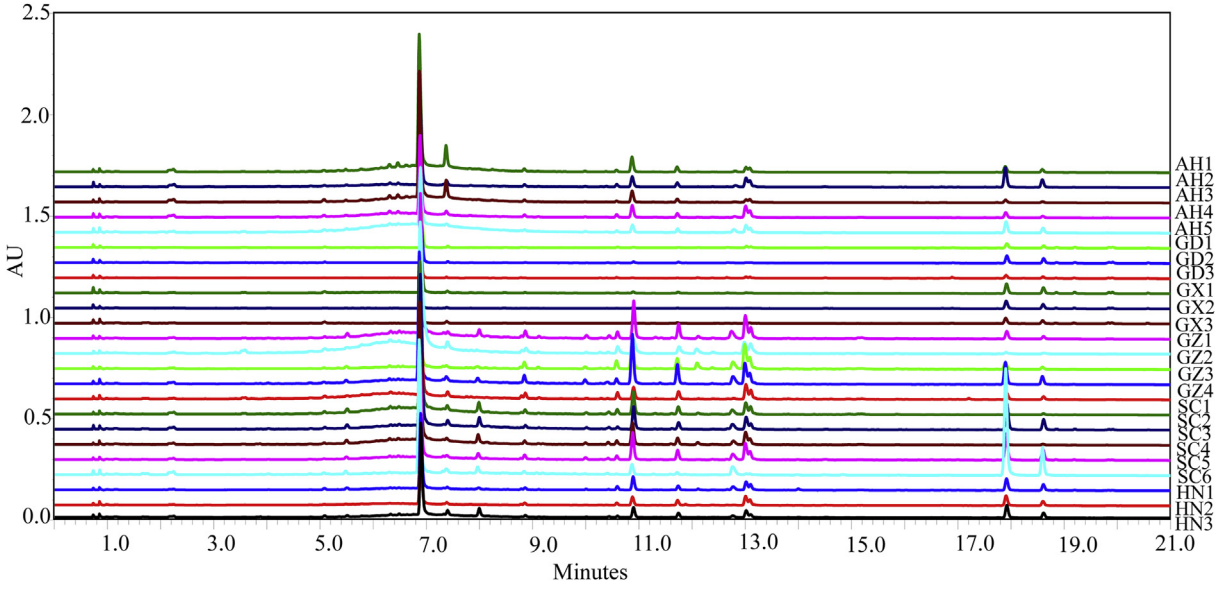


Figure 1 – UPLC–PDA chromatographic fingerprints at 260 nm of 24 samples from six different geographical regions as listed in Table 1. PDA = photodiode array detection; UPLC = ultra-high-performance liquid chromatograph.

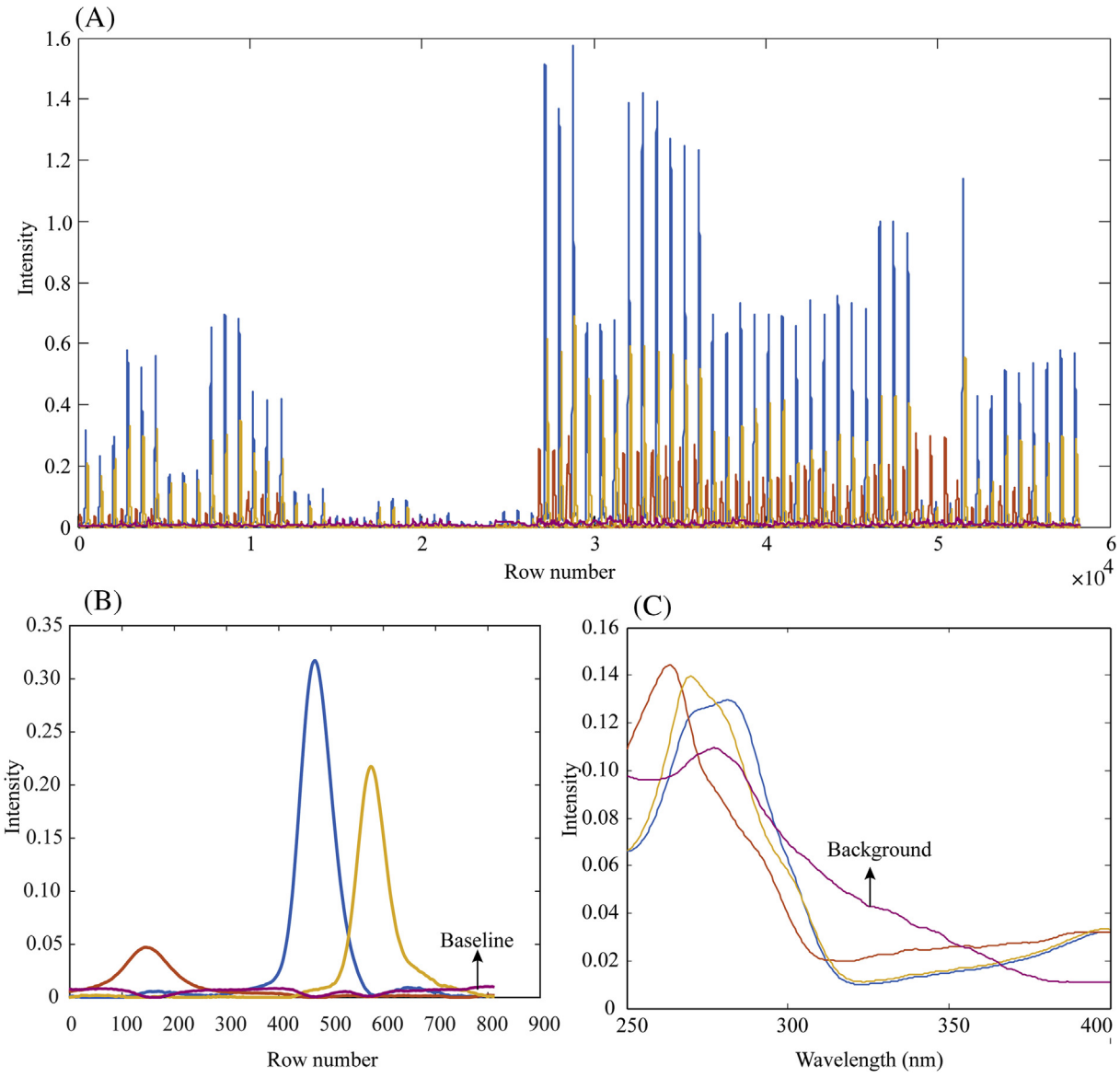


Figure 2 – MCR–ALS results of chromatographic segment k. (A) The resolved elution profiles of augmented MCR–ALS, (B) pure elution profile of sample AH11, and (C) the resolved spectral profiles. ALS = alternating least squares; MCR = multivariate curve resolution.

classification to show similarities and differences of the chromatographic fingerprints.

The 72 (samples) × 22 (variables) data matrix was submitted to PCA, and autoscaling was selected as a preprocess step prior to analysis. The PC1 versus PC2 plot shared 69.46% data variance (PC1 = 52.93%, PC2 = 16.53%). Group separation can be clearly seen in Figure 3, and samples fell into four clusters. Samples from Guangdong and Guangxi were located near to one another, and could be regarded as one cluster (Cluster II). The samples from Anhui and Henan were also close to one another, and could be considered as another cluster (Cluster I). The Sichuan and Guizhou samples constituted two independent clusters (Clusters IV and III, respectively). Guangdong and Guangxi are located in the southern most mainland of China with a subtropical monsoon climate; Guizhou is situated in the southwest China mountain plateau, and has a subtropical humid monsoon climate; Henan and Anhui are located near to each other in East Central China, and have a subtropical to warm temperate transition subhumid mountain climate; Sichuan is located in the southwest mainland of China, and has a subtropical monsoon climate. The cluster results may be related to the geographical locations and climates of the source areas to a large extent.

Ward's method was also applied, not only for a better classification, but also for identification of the most important of the 22 vectors. Figure 4 shows the classification results achieved by Ward's method cluster analysis. The dendrogram of 72 PMR samples is shown in Figure 4A, in which the linkage value of 27.92 was selected as the threshold. The samples could clearly be divided into four independent clusters, which was consistent with the results from PCA. Figure 4B shows the variance-weighted distances between the cluster centers of

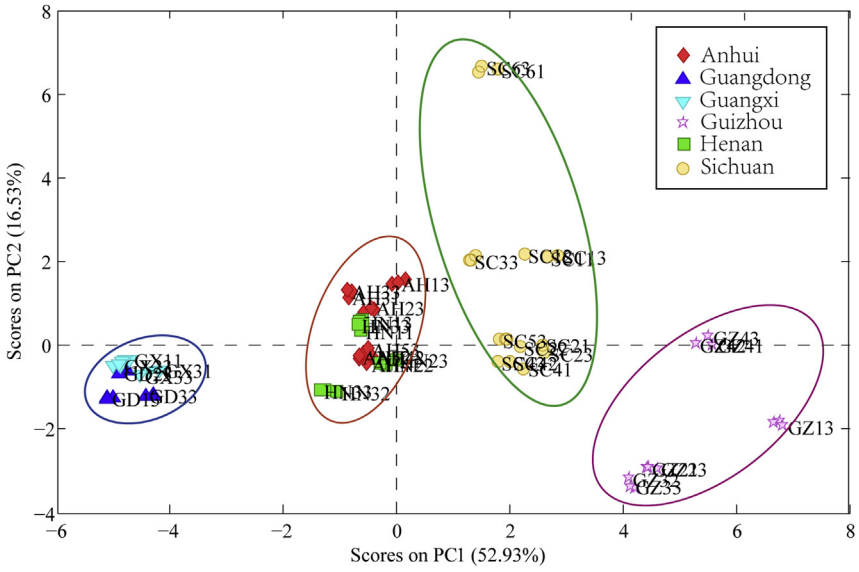


Figure 3 – Two-dimensional PCA score plot (PC1 vs. PC2) of all chromatographic fingerprints of Polygoni Multiflori Radix samples as listed in Table 1. PCA = principal component analysis.

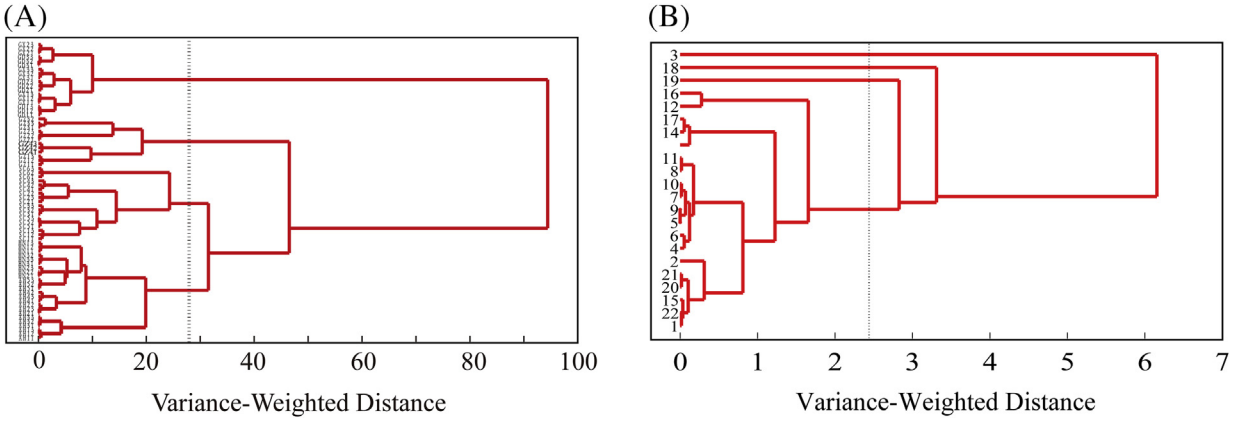


Figure 4 – The variance-weighted distances between cluster centers of (A) 72 Polygoni Multiflori Radix samples and (B) 22 components obtained from Ward's method.

the 22 vectors. Five vectors (3, 12, 16, 18, and 19) were selected as the main ones by choosing a linkage value of 2.245. Therefore, 72 PMR samples were classified into four clusters, and five most important vectors were selected to discriminate these clusters.

A CP-ANN is a supervised pattern-recognition method, which was applied to investigate the effects of the main vectors (3, 12, 16, 18, and 19) in different samples. The new matrix (72 × 5) contains the relative species concentration of the five main vectors. The samples were first divided into four classes of I, II, III, and IV, according to the results of PCA and Ward's method. The new matrix served as an input to the Kohonen and CP-ANN toolbox. Values of 1, 2, 3, and 4 were used to represent the assigned classes I, II, III, and IV, respectively. The Kohonen map is composed of a grid of N^2 neurons, where N represents the neurons for each side of the squared space [26]. The optimum model was achieved, in which the network consisted of 64 neurons (8 × 8) and the number of epochs was 50. The results of CP-ANN analysis with the optimum parameters are shown in Figure 5. Figure 5A shows the distribution of samples on the Kohonen map and Figure 5B shows the assigned classes of the samples. The 72 samples occupy different neurons of the Kohonen map (Figure 5). Replicate samples are located in the same or in an adjacent neuron, which indicates good repeatability of the experiment. There was good agreement among the results of CP-ANN analysis, PCA scores plot (Figure 3), and Ward's method (Figure 4). For example, SC21, SC22, and SC23 are located near to each other and next to SC41, SC42, and SC43, and far from SC11, SC12, and SC13, which shows the good agreement among the different classification methods.

The weights of the five main vectors in each class obtained from CP-ANN analysis are shown in Figure 6. As can be seen, Variable 3 is the most important for Class I; Variables 3, 12, and 16 are the most important for Class III; and Variables 3, 12, and 19 are the most important for Class IV. Therefore, Variable 16 was the most discriminant variable for Class III, and Variable 19 was the most discriminant variable for Class IV.

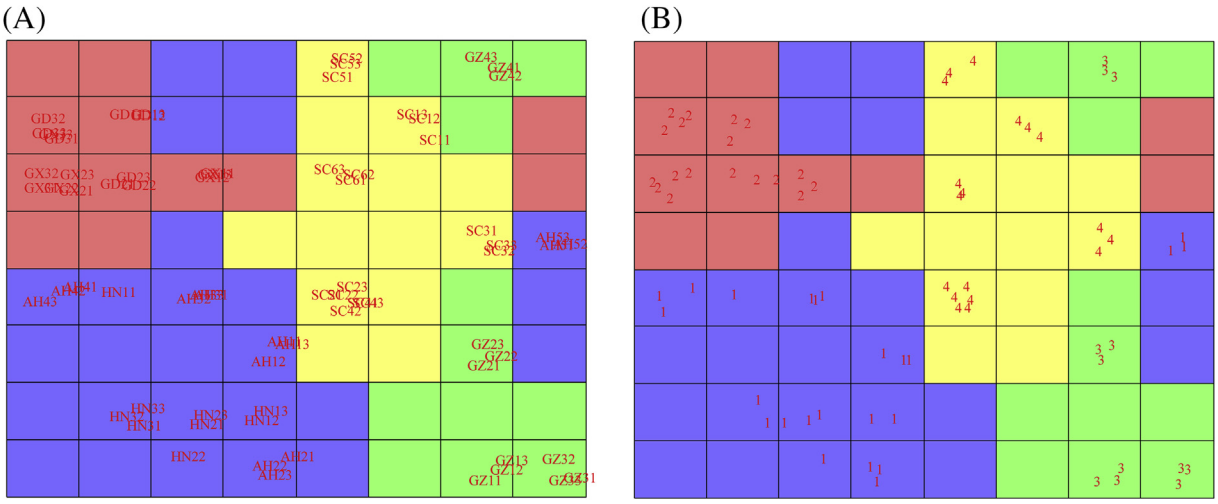


Figure 5 – Kohonen map of 72 samples. (A) Distribution of 72 Polygoni Multiflori Radix samples on the map and (B) assigned group distribution on the map.

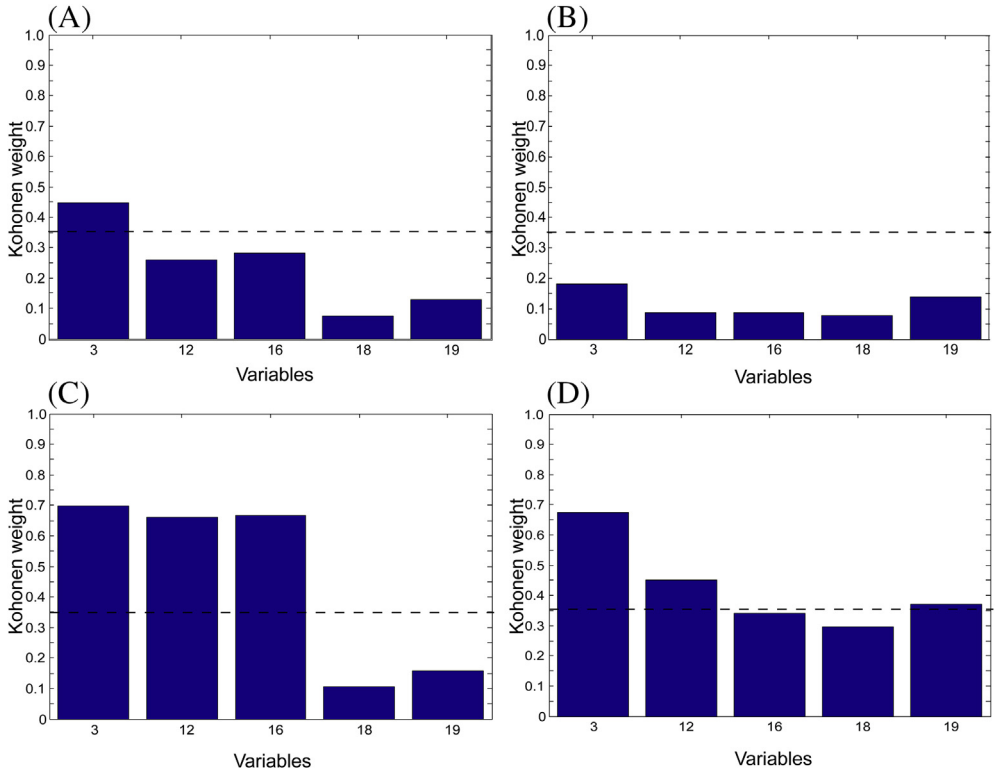


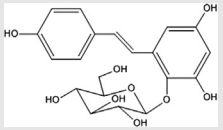
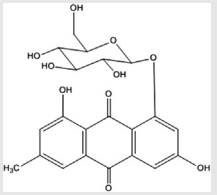
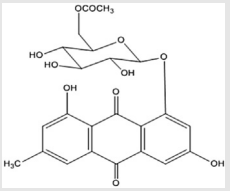
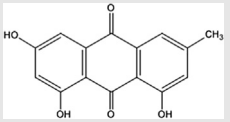
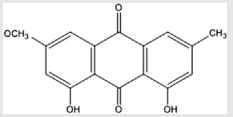
Figure 6 – The weights of five main vectors in each class obtained from CP-ANN analysis. (A) Cluster I, (B) Cluster II, (C) Cluster III, and (D) Cluster IV. CP-ANN = counter-propagation artificial neural network.

Table 2 – Five chemical markers identified in Polygoni Multiflori Radix by UPLC–Q-TOF-MS in negative-ion mode.

Peak no.	t_R (min)	*m/z*	Molecular formula	Identified marker
3	6.89	405, 243	$C_{20}H_{22}O_9$	2,3,5,4′-Tetrahydroxy-stilbene-2-O-β-D-glucoside
12	10.88	431, 269	$C_{21}H_{20}O_{10}$	Emodin-8-O-β-D-glucopyranoside
16	12.87	473, 431, 269	$C_{23}H_{22}O_{11}$	Emodin-8-O-(6′-O-acetyl)-β-D-glucopyranoside
18	17.78	269, 270	$C_{15}H_{10}O_5$	Emodin
19	18.52	283	$C_{16}H_{12}O_5$	Physcion

Q-TOF-MS = time-of-flight mass spectrometer; UPLC = ultra-high-performance liquid chromatograph.

Table 3 – Biological and pharmacological properties of chemical markers in Polygoni Multiflori Radix.

Peak no.	Chemical compound	Chemical structure	Biological and pharmacological properties
3	2,3,5,4′-Tetrahydroxy-stilbene-2-O-β-D-glucoside		Antioxidant activities, hepatoprotective effect, lipid regulation effects, prevention and treatment of Alzheimer's disease, neuroprotective effect
12	Emodin-8-O-β-D-glucoside		Antioxidant activities, antitumor activity, effect of improving memory, antihuman immunodeficiency virus activities
16	Emodin-8-O-(6′-O-acetyl)-β-D-glucopyranoside		Not reported
18	Emodin		Anti-inflammatory, antitumor activity, antibacterial activity, immunologic enhancement, hepatoprotective effect, myocardial protection
19	Physcion		Antibacterial activity

3.4. *Identification of significant components*

The five chemical markers were identified by pure spectral profiles obtained from MCR–ALS, MS information obtained from UPLC–Q-TOF-MS, and the retention times obtained from standards. The five chemical marker components (3, 12, 16, 18, and 19) were identified as 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside, emodin-8-O-β-D-glucopyranoside, emodin-8-O-(6′-O-acetyl)-β-D-glucopyranoside, emodin, and physcion, respectively (Table 2) [30,31]. It is interesting that the five significant components were present at high concentrations in PMR.

3.5. *Biological and pharmaceutical activities of chemical markers*

Table 3 lists the information of chemical markers, including the chemical structure and biological and pharmaceutical properties in PMR samples from different geographical origins [32–37]. As these valuable compounds can be found at high concentrations in PMR, the root can be considered as a valuable herbal medicine and functional food.

A comprehensive method can be established by using these five markers as standards to evaluate the quality of PMR. In addition, unknown samples from five of the different regions studied could be classified according to the concentrations of these five chemical markers. Nevertheless, many samples were still needed to quantify these chemical markers.

4. Conclusions

A chemometric strategy based on a combination of MCR–ALS and multivariate classification methods has been developed to comprehensively classify the UPLC–photodiode array detection fingerprints of PMR. The MCR–ALS model provided a solution to common chromatographic problems, including the background contribution, baseline contribution, peak overlap, and so on. A total of 22 chemical components were obtained from the MCR–ALS model. PCA and Ward's method were applied to classify the samples. The samples were separated into four clusters, and five chemical markers were identified for discrimination. In addition, a CP-ANN has been applied to explore the effect of these five chemical markers in the different samples. The markers provide a more comprehensive way to evaluate the quality of PMR. The results indicated that the developed chemometric strategy was accurate and showed good prospects for the analysis of natural samples.

Conflicts of interest

The authors declare no conflicts of interest.

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