International Journal of Chinese Medicine
2020; 4(1): 1-11
http://www.sciencepublishinggroup.com/j/ijcm
doi: 10.11648/j.ijcm.20200401.11
ISSN: 2578-9465 (Print); ISSN: 2578-9473 (Online)

Combination of HPLC Fingerprint and QAMS as a New Analytical Approach for Determination of Bufadienolides in *Bufonis Venenum*

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To cite this article:

Yi Luo, Cejia Liu, Shengci Fan, Zhaoqing Meng, Fei He. Combination of HPLC Fingerprint and QAMS as a New Analytical Approach for Determination of Bufadienolides in *Bufonis Venenum*. *International Journal of Chinese Medicine*. Vol. 4, No. 1, 2020, pp. 1-11.
doi: 10.11648/j.ijcm.20200401.11

Received: November 28, 2019; **Accepted**: December 18, 2019; **Published**: January 21, 2020

Abstract: The present study presents an effective and comprehensive evaluation method for assessing the quality of *Bufonis Venenum*, based on analysis of high performance liquid chromatography (HPLC) fingerprints in combination with similarity analysis, hierarchical cluster analysis (HCA), principal component analysis (PCA) and a quantitative analysis multi-components by single marker (QAMS) method. Nine common peaks identified by fingerprint detection results of 10 batches of samples were collected and used for the similarity analysis, HCA, PCA and QAMS analysis. These methods drew a similar conclusion that the quantitative analysis multi-components by single marker (QAMS) method 41 *Bufonis Venenum* samples were categorized into four main groups by HCA and PCA, and the majority of the samples with similar ingredients were mainly concentrated in Shandong area. When QAMS method was compared with the external standard method (ESM), it was feasible to evaluate the quality of *Bufonis Venenum* by the values of relative correction factors (RCFs) from cinobufagin (the internal reference) versus arenobufagin, bufalin, bufotalin, resibufogenin and telocinobufogin. In conclusion, these methods were successfully applied to identification of the origin and evaluation the quality of *Bufonis Venenum*. Therefore, these evaluation methods are promising to be widely applied in the quality control of Traditional Chinese Medicines (TCMs).

Keywords: QAMS, Fingerprint, Bufadienolides, *Bufonis Venenum*, Method

1. Introduction

Bufonis Venenum is a dry secretion derived from the parotoid glands, and skin of two species of toads, *Bufo bufo gargarizans* Cantor and *B. melanostrictus* Schneider [1, 2]. It is also known as Chansu in China and Senso in Japan. The earliest record of Chansu appeared in Tang Dynasty (618-907) [3], and today there are 88 known formulations within containing *Bufonis Venenum*, including Hou-Zheng-Wan [4] and Xiong-Dan-Jiu-Xin-Wan [5]. *Bufonis Venenum* has long been used in traditional Chinese medicine, as a treatment for heart failure, tumors, sores, and pains in clinical settings. The beneficial properties of *Bufonis Venenum* may be arributable to several of its active chemical constituents, primarily bufadienolides and indole alkaloids [1, 2, 6]. To date, according to the references reported, 71 kinds of bufadienolide have been described, of which cinobufagin, resibufogenin, telocinobufogin, arenobufagin, bufalin and bufotalin are typically the most abundant. Moreover, these six compounds are known to possess cardiac function and antitumor activities [7-9]. Bufalin is also known to possess anti-inflammatory and anesthetic properties [6, 10].

According to the Chinese Pharmacopoeia (2015 ed.), only toads belonging to the *B. gargarizans* and *B. melanositicuts* species may be used for the preparation of *Bufonis Venenum*. In recent years, with the increase of the demand for *Bufonis Venenum* and the price boosting, leading to the mixture of the origin of *Bufonis Venenum* and the widespread adulteration and jumbled. For now, there is no method to detect both the authenticity and the origin. Further, only two compounds, cinobufagin and reasibufogenin, are to be quantified as the control marker in the Chinese Pharmacopoeia (2015 ed.). The

existing quality control standards are insufficient to evaluate and identify the quality of *Bufonis Venenum*. We need higher quality standards to evaluate quality.

In recent years, chromatographic fingerprint analysis has been accepted as a strategy for quality assessment of herbal medicines and preparations by the WHO, the FDA and the State Food and Drug Administration (SFDA) of China [11, 12]. Application of such methods has revealed the presence of substitutes and adulterants in a number of traditional medicinal materials, and so is a valuable approach for the quality control of TCMs [13, 14].

The external standard method (ESM) has been the most commonly employed approach for the qualitative and quantitative analysis of components in TCM. However, in recent years, quality control and evaluation of TCMs by ESM has become challenging in some cases due to the high cost of some reference standards, the diversity and complexity of TCMs, the instability of detection and many other uncertainties [15]. In order to tackle this issue, a quantitative analysis multi-components by single marker (QAMS) method has been raised [16]. It can not only greatly reduce the detection time and the cost of the experiment, but improve the practicability of the method and control the quality of TCMs more effectively and comprehensively.

Therefore, a more comprehensive and effective chemical analysis method is needed. In the present study, we attempt to establish a method that could discern the false from the genuine and distinguish the place of origin. We try to combine fingerprint and QAMS detect the *Bufonis Venenum* samples for quality standard improvement, and similarity analysis, hierarchical cluster analysis (HCA), principal component analysis (PCA) for distinguishing the origin. This method would be the first to identify both authenticity and origin.

2. Experimental

2.1. Instruments

The chromatographic system was controlled by an Agilent 1260 series (Agilent, USA) HPLC chromatograph with quaternary pump, a 20 μL sample loop and a diode array detector (DAD). The SHB-B95A-type (Zhengzhou, China) vacuum pump and KQ-300DE-type heating reflector (Kunshan, China) were used for sample extraction.

2.2. Materials

We collected toads' samples from five geographical regions and three origins. Then, derive white secretions from the parotid glands of toads. Dry secretions at 60°C, content of water were not more than 13.0%. Finally, we got forty one *Bufonis Venenum* samples. Samples were stored in dryer at 25°C. The information of samples has been listed in Table 1.

Table 1. *Different species and geographical locations of 41 Bufonis Venenum samples in China.*

No.	Regions	Origins	No.	Regions	Origins	No.	Regions	Origins
1	Shandong	Bufo bufo gargarizans Cantor	15	Shandong	Bufo bufo gargarizans Cantor	29	Anhui	Bufo melanostictus Schneider
2	Sichuan	Bufo bufo gargarizans Cantor	16	Shandong	Bufo bufo gargarizans Cantor	30	Anhui	Bufo melanostictus Schneider
3	Shandong	Bufo bufo gargarizans Cantor	17	Heibei	Bufo bufo gargarizans Cantor	31	Shandong	Bufo bufo gargarizans Cantor
4	Heibei	Bufo bufo gargarizans Cantor	18	Sichuan	Bufo bufo gargarizans Cantor	32	Sichuan	Bufo bufo gargarizans Cantor
5	Heibei	Bufo bufo gargarizans Cantor	19	Shandong	Bufo bufo gargarizans Cantor	33	Shandong	Bufo bufo gargarizans Cantor
6	Shandong	Bufo bufo gargarizans Cantor	20	Heibei	Bufo bufo gargarizans Cantor	34	Jiangsu	Bufo melanostictus Schneider
7	Shandong	Bufo bufo gargarizans Cantor	21	Heibei	Bufo bufo gargarizans Cantor	35	Sichuan	Bufo bufo gargarizans Cantor
8	Heibei	Bufo bufo gargarizans Cantor	22	Sichuan	Bufo bufo gargarizans Cantor	36	Jiangsu	Bufo melanostictus Schneider
9	Shandong	Bufo bufo gargarizans Cantor	23	Heibei	Bufo bufo gargarizans Cantor	37	Jiangsu	Bufo melanostictus Schneider
10	Heibei	Bufo bufo gargarizans Cantor	24	Anhui	Bufo melanostictus Schneider	38	Jiangsu	Bufo melanostictus Schneider
11	Heibei	Bufo bufo gargarizans Cantor	25	Anhui	Bufo melanostictus Schneider	39	Sichuan	Bufo raddei Strauch

2.3. Chemical Reagents and Standards

Methanol was provided by Tianjin Fuyu Fine Chemical Co., Ltd., China, and acetonitrile and formic acid for HPLC were purchased from Merck & Co., Inc, USA. In terms of standards, arenobufagin (No. 20180945), bufotalin (No. 20180314) and telocinobufogin (No. 20181089) were purchased from Wan Xiang Heng Yuan biological technology Co., Ltd (Tianjin, China), while bufalin (No. 111981-201501), cinobufagin (No. 110803-201406) and resibufogenin (No. 110718-201108) were purchased from National Instituted for Food and Drug Control (Beijing, China). Purity of these ingredients was greater than 98% as determined by HPLC.

2.4. Preparation of Sample Solution

The dried *Bufonis Venenum* sample 25mg was accurately weighed and extracted with 20ml methanol by heating

reflector for 1h. Any weight reduction was countered by the addition of more methanol as required. The solution was then filtered by 0.22μm polyvinylidene fluoride microporous filter. Finally, the resultant filtrate was stored at 4°C pending analysis.

2.5. Reference Solution Preparation

Stock solutions of each reference standard at a concentration of 1mg mL^{-1} were prepared by dissolving an accurately weighed amount of each reference substance (arenobufagin, bufalin, bufotalin, cinobufagin, resibufogenin and telocinobufogin) in methanol, and were stored at 4°C. The stock solutions were then diluted to establish the calibration curves based on five appropriate concentrations with the ranges of 0.011-0.360 mg mL^{-1} for arenobufagin, 0.002-0.071 mg mL^{-1} for telocinobufogin, 0.007-0.238 mg mL^{-1} for bufotalin, 0.006-0.202 mg mL^{-1} for bufalin, 0.013-0.409 mg mL^{-1} for cinobufagin, and 0.008-0.245 mg mL^{-1} for resibufogenin.

2.6. Chromatographic Procedures

An Agilent ZORBAX SB C18 Inertsil (Agilent, USA) 5μm column (250mm×4.6mm) was used for chromatographic separation of *Bufonis Venenum* sample. An Agilent ZORBAX Bonus-RP C18 5μm column (250mm×4.6mm) and Alltima C18 (Alltech, USA) 5μm column (250mm×4.6mm) were used for system suitability test. The mobile phase consisted of 0.1% formic acid in deionized water (A) and acetonitrile (B). The gradient elution was as follows: 0-20min, 80-70% A; 20-25min, 70-65% A; 25-40min, 65% A; 40-50min, 65-50% A; 50-60min, 50-40% A. The UV detection wavelength was 296nm. The flow rate was 0.7 mL min^{-1}, and the injection volume was 20 μL. The column temperature was kept at 25°C throughout.

The obtained chromatographic peaks were identified by comparing the retention time, the on-line UV spectra and chemical reference substances.

2.7. Data Analysis

All data were analyzed and using the Similarity Evaluation System for chromatographic fingerprint of TCMs (CASE, version 2004A) recommended by the SFDA of China for evaluating similarities of chromatographic profiles of TCMs. The similarity among different chromatograms was quantified by calculating the correlative coefficient and/or cosine value of the vectorial angle. Hierarchical clustering analysis (HCA) was performed based on Squared Euclidean Distance to distinguish toad distribution using the SPSS software. Additionally, principal component analysis (PCA) was used to visualize the data in 2D space. Simultaneously, to validate the feasibility of QAMS by comparing with ESM, cinobufagin was used as an internal reference in QAMS to determine other five active components in 41 *Bufonis Venenum* samples.

3. Results and Discussion

3.1. Chromatograph Optimization

To obtain an optimal chromatographic fingerprint, it was important to select an appropriate extraction method and HPLC parameters. Our results revealed that extracting 25mg of *Bufonis Venenum* with 20ml methanol via heating reflector for 1h yielded high-quality filtrate, and gave the most reproducible chromatographic fingerprint. Furthermore, the chromatographic conditions detailed in section 2.6 were demonstrated to be the most suitable.

3.2. Method Validation

To validate our approach, one sample (No. 2) was randomly selected as the example. The precision test was performed by consecutively injecting the same sample solution six times. The stability test was performed by six injections of the same sample solution for at 0h, 2h, 4h, 8h, 12h and 24h at room temperature (25±2°C). The precision and stability were determined by analyzing relative retention time (RRT) and relative peak area (RPA) of compounds (No. peaks: 2, 3, 4, 6, 7 and 8) of six chromatogram profiles from *Bufonis Venenum* samples, respectively. The results of precision and stability analyses showed that the relative standard deviation (RSD) of the RRT and RPA were both less than 3%. Furthermore, the repeatability test was performed via six injections of independently prepared samples (derived from biological sample No. 2), with the results exhibiting that the RSD of the RRT and RPA of compounds (No. peaks: 2, 3, 4, 6, 7 and 8) were between 0.00- 0.77% and 0.00- 3.22% (n=6), respectively. The recovery rates ranged from 98.24-100.79% with RSD values lower than 3%. Together, these results (Tables 2 and 3) suggested that these methods were effective and reliable.

Table 2. *Analytical results of precision, repeatability and stability tests.*

Analytes	Precision (n=6)				Repeatability (n=6)				Stability (n=6)			
	RRT	RSD%	RPA	RSD%	RRT	RSD%	RPA	RSD%	RRT	RSD%	RPA	RSD%
Arenobufagin	0.40	0.80	1.07	0.71	0.40	0.68	1.13	2.46	0.40	1.64	0.89	0.46
Telocinobufagin	0.60	0.62	0.26	0.68	0.60	0.75	0.20	2.15	0.60	1.05	0.16	4.24
Bufotalin	0.65	0.65	0.46	0.64	0.65	0.77	0.56	2.41	0.65	1.12	0.44	0.41
Bufalin	0.89	0.26	0.51	0.72	0.89	0.44	0.62	3.22	0.89	0.50	0.50	1.47
Cinobufotalin	1.00	0.13	1.00	0.72	1.00	0.30	1.00	3.10	1.00	0.28	1.00	0.17
Resibufogenin	1.03	0.12	0.53	0.81	1.03	0.34	0.63	3.09	1.03	0.28	0.51	0.72

Table 3. *Analytical results of recovery tests of the added standard sample.*

Analytes	%	RSD%
Arenobufagin	98.24	2.37
Telocinobufagin	99.44	2.25
Bufotalin	98.41	2.48
Bufalin	98.37	2.26
Cinobufotalin	99.15	2.50
Resibufogenin	100.79	2.14

3.3. Calibration Curves

The calibration curves of the six standard substances were constructed using appropriate concentrations for HPLC analysis. The calibration curve (y=ax+b) was plotted as base on relative peak areas (y) versus the concentration of analytes (μg mL^{-1}, x). Furthermore, linearity, limits of detection (LODs) and limits of quantification (LOQs) of each individual standards were assessed using calibration curves. The results (Table 4) showed that linearity, LODs and LOQs for each investigated compound were stable and the obtained calibration curves could be applied to QAMS analysis.

Table 4. *Calibration curves, LOQs and LODs of HPLC method for determination of arenobufagin, bufalin, bufotalin, cinobufagin, resibufogenin and telocinobufogin.*

Analytes	Test range (mg mL^{-1})	Calibration curve	Correlation coefficient (R^2)	LOD (mg mL^{-1})	LOQ (mg mL^{-1})
Arenobufagin	0.010-0.557	y=13748x+236.06	0.9999	0.008	0.011
Bufalin	0.002-0.090	y=9980.3x+34.599	0.9996	0.001	0.003
Bufotalin	0.005-0.501	y=8447.2x+96.422	0.9998	0.003	0.005
Cinobufagin	0.005-0.478	y=11592x+114.78	0.9990	0.003	0.007
Resibufogenin	0.008-0.914	y=15154x+286.40	0.9998	0.006	0.012
Telocinobufogin	0.005-0.639	y=8685.8x+103.36	0.9998	0.004	0.007

Note: LODs: limits of detection; LOQ: limits of quantitation. In the regression equation y=ax+b, y refers to the peak area, x to the concentration of the arenobufagin, bufalin, bufotalin, cinobufagin, resibufogenin and telocinobufogin (mg mL^{-1}), respectively, and R^2 is the coefficient of the equation.

3.4. HPLC Fingerprint Analysis

To perform HPLC fingerprint analysis, the standardized fingerprint chromatograms of all *Bufonis Venenum* samples were obtained. This process included the selection of "common peaks" of fingerprint chromatograms and the normalization of retention times of all the common peaks. Here, the fingerprint chromatograms from *Bufonis Venenum* were analyzed (Figure 1). The RRT and RPA of 8 characteristic peaks were selected for establishing the common pattern, respectively (Figure 2). In addition, according to the RRT of the reference fingerprint, peaks 2, 3, 4, 6, 7 and 8 were identified as arenobufagin, telocinobufagin, bufotalin, bufalin, cinobufagin and resibufogenin, respectively (Figure 3).

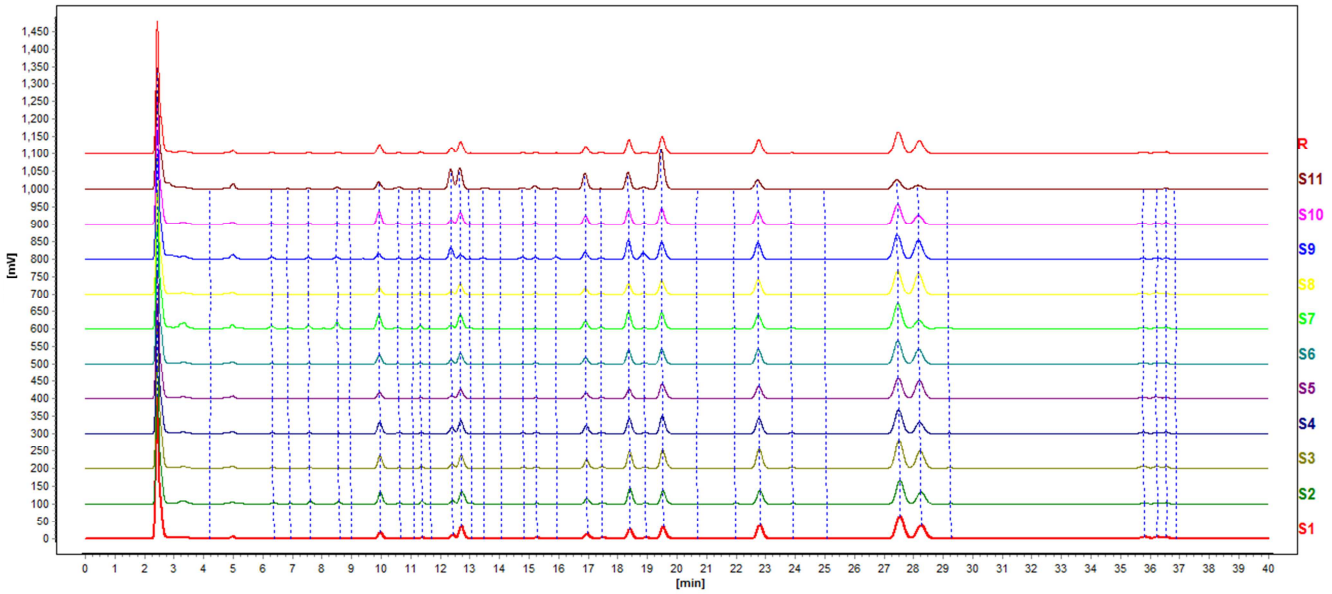

Figure 1. *HPLC fingerprints for 10 Bufonis Venenum samples.*

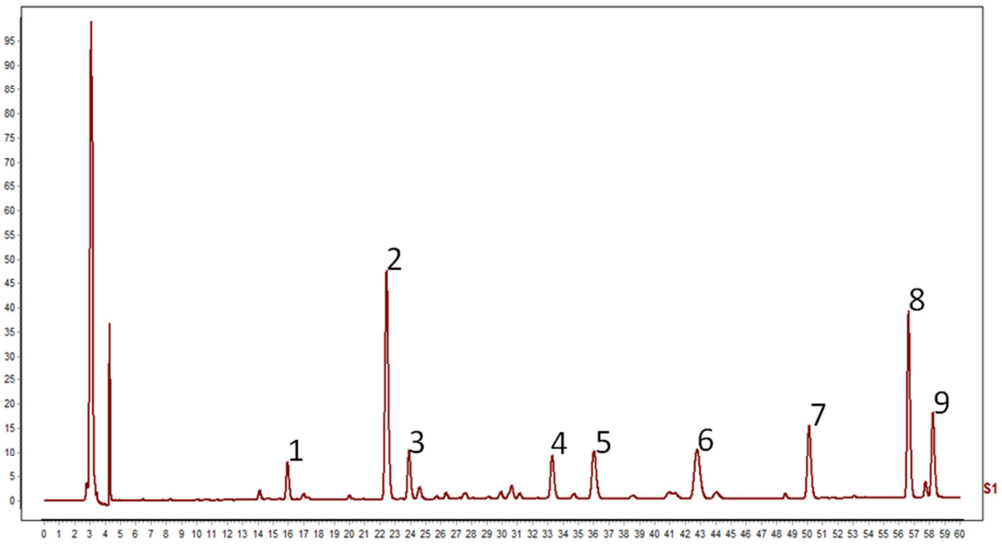

Figure 2. *Common model fingerprints of Bufonis Venenum samples (S1). Identified peaks: (2) arenobufagin; (4) telocinobufagin; (5) bufotalin; (7) bufalin; (8) cinobufagin; (9) Resibufogenin.*

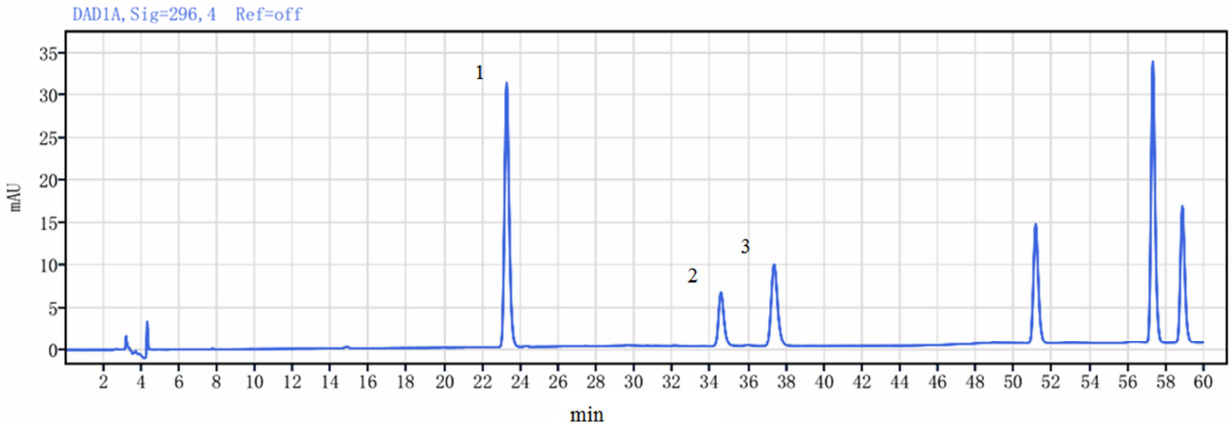

Figure 3. *Chromatogram of reference standards. 1. arenobufagin; 2. telocinobufagin; 3. bufotalin; 4. bufalin; 5. cinobufagin; 6. resibufogenin.*

3.5. Similarity Analysis

The results are summarized in Table 5. The similarity values among of the same location were very close. For example, the similarity values of *Bufonis Venenum* samples from Shandong area were more than 0.800 in most cases, indicating a high level of similarity. The samples from Sichuan area had the lowest similarity values. Together, our results showed that the origin of samples influenced the quality of *Bufonis Venenum*.

Table 5. *The similarities index of fingerprint chromatogram of 41 Bufonis Venenum samples.*

No.	Similarities	No.	Similarities	No.	Similarities
1	0.894	15	0.910	29	0.442
2	0.926	16	0.898	30	0.444
3	0.887	17	0.921	31	0.575
4	0.918	18	0.926	32	0.911
5	0.920	19	0.898	33	0.813
6	0.919	20	0.917	34	0.656
7	0.902	21	0.924	35	0.750
8	0.922	22	0.904	36	0.886
9	0.893	23	0.929	37	0.269
10	0.922	24	0.478	38	0.348
11	0.924	25	0.494	39	0.537
12	0.898	26	0.978	40	0.878
13	0.468	27	0.886	41	0.258
14	0.919	28	0.895		

3.6. Hierachical Cluster Analysis (HCA)

The clustering analysis of 41 *Bufonis Venenum* samples was carried out using the SPSS Statistics 19.0 software. HCA can efficiently produce a qualitative and quantitative representation of the original experimental results through statistical organization and graphical display distinguishing different species in TCMs. The dendrograms (Figure 4) and icicles (Figure 5) were constructed according to HCA based on the squared euclidean distance. When the 9 characteristic peaks were used as the clustering variable, the 42 samples were categorized into four groups containing 23 samples (Group 1), 16 samples (Group 2), No. 41 and No. 39. Using the same squared Euclidean space, the groups were further divided into subgroup 1a (No. 4, 5, 8, 10, 11, 13, 14, 17, 20, 21 and 23), subgroup 1b (No. 1, 3, 6, 7, 9, 12, 15, 16, 19, 31, 33 and 40), subgroup 2a (No. 24, 25, 26, 27, 28, 29, 30, 34, 36, 37 and 38) and subgroup 2b (No. 2, 18, 22, 32 and 35). According to table 1 information of 41 *Bufonis Venenum* samples, samples from the same geographical region were cluster into one subgroup. Subgroup 1a samples were all sampled from Hebei, subgroup 1b from Shandong, subgroup 2a from Anhui and Jiangsu, subgroup 2b from Sichuan. Since Anhui and Jiangsu are adjacent, they are not distinguished. Although No. 41 was also sampled from Sichan, but its origin is Bufo raddei Strauch, which is different from Group1 and Group2. No. 39 is fake sample, so it is a separate cluster.

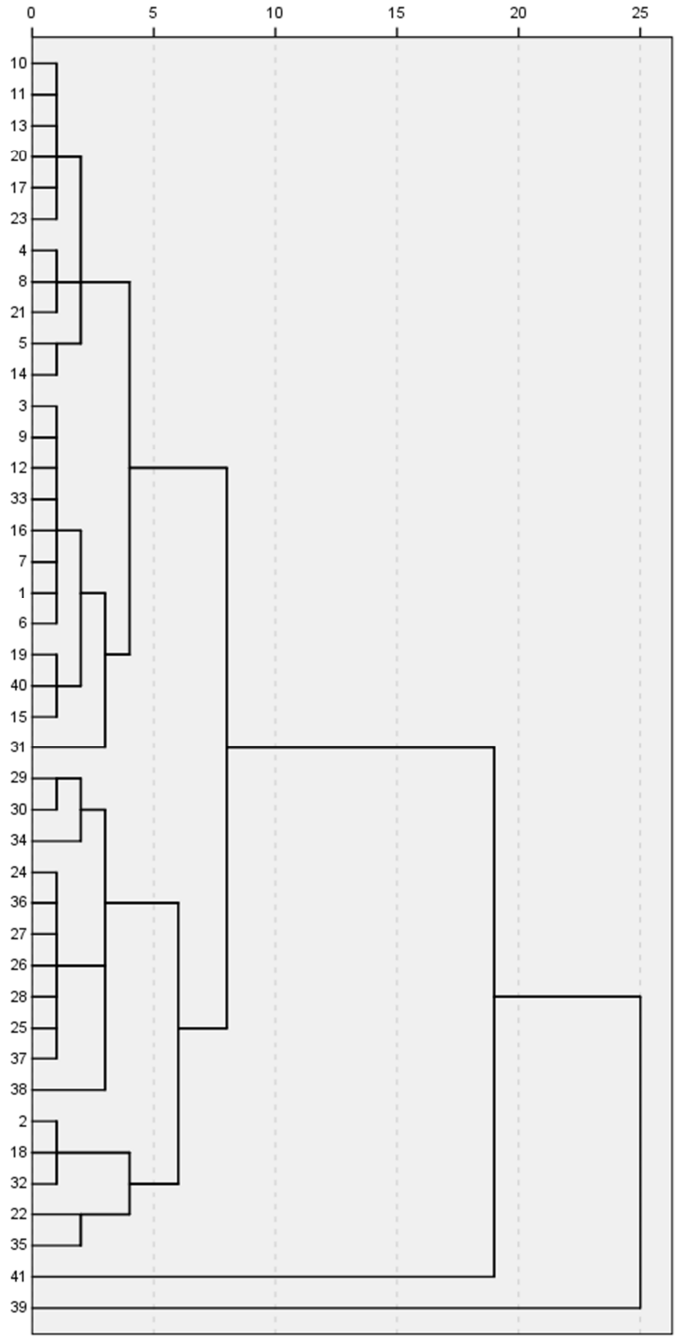

Figure 4. *Clustering analysis graph of 41 Bufonis Venenum samples.*

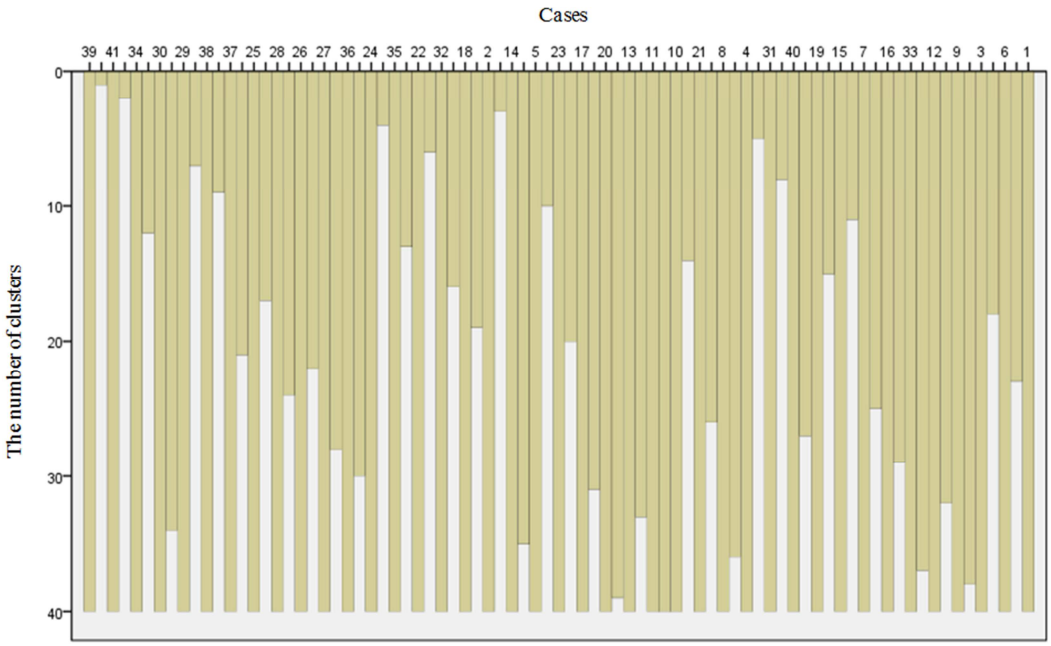

Figure 5. *Icicles for the 41 Bufonis Venenum smaples.*

To a certain extent, the results of HCA showed were consistent with those of the similarity analysis. Samples from same geographical region have the similar similarity. According to the results of HCA and similarity analysis, besides of geographical region as the identification factors, origins may be another factor.

3.7. Principal Component Analysis (PCA)

Cumulatively, PC1 and PC2 accounted for 75.548% of total variance (PC1, 43.826%; PC2, 31.722%). Thus, to cluster all the samples, PC1 and PC2 were used for the score plots (Figure 6). It showed that 41 samples were categorized into six groups: cluster 1 (No. 4, 5, 8, 10, 11, 13, 14, 17, 20, 21 and 23), cluster 2 (No. 1, 3, 6, 7, 9, 12, 15, 16, 19, 31, 33 and 40), cluster 3 (No. 24, 25, 26, 27, 28, 29, 30, 34, 36, 37 and 38), cluster 4 (No. 2, 18, 22, 32 and 35), No. 39 and No. 41. According to PCA results, four subgroups, No. 39 and No. 41 were separated by different geographical region and different origins. Samples from same geographical region were in one cluster. Cluster1 contains samples from Hebei, cluster2 from Shandong, cluster3 from Anhui and Jiangsu, and cluster4 from Sichuan. No. 39 and No. 41 were in separated clusters. The origins of cluster1, cluster2 and cluster3 were Bufo bufo gargarizans Cantor, and cluster 4 was Bufo melanostictus Schneider, No. 39 was Bufo raddei Strauch and No. 41 was fake sample. We could distinguish the different geographical region and different origins according to the PCA results.

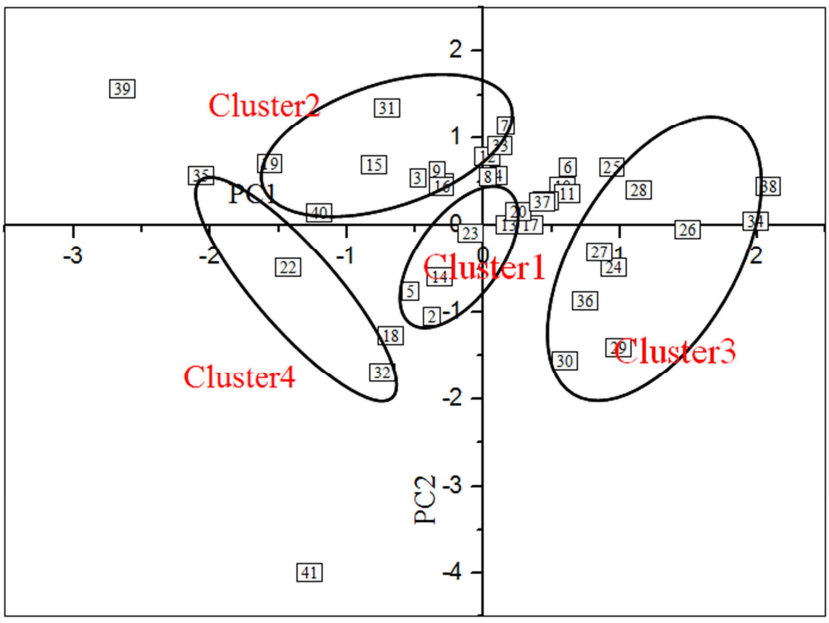

Figure 6. *The score plots obtained from PCA analysis of 41 Bufonis Venenum samples.*

3.8. Quantitative Analysis Multi-components by Single Marker (QAMS)

Due to its availability, stability and low price, cinobufagin was selected as the internal standard. To validate the feasibility of QAMS, the classic Estandard Standard Method (ESM) approach for the determination of the other five standards (arenobufagin, telocinobufagin, bufotalin, Bufalin and resibufogenin) was also carried out simulataneously. Thus, the five investigated components of all 41 *Bufonis Venenum* samples were determined using both the ESM and QAMS methods.

The RCFs were calculated using the calibration curves. The results are shown in Table 6. The process of quantitative determination of *Bufonis Venenum* samples involved the following three steps: (1) Cinobufagin was chosen as the internal standard and RCFs were obtained for arenobufagin, telocinobufagin, bufotalin, bufalin and resibufogenin by calculating the ratios of the slopes of their calibration equations to that of cinobufagin. (2) The contents of six standard solutions (arenobufagin, telocinobufagin, bufotalin, bufalin, cinobufagin and resibufogenin) were directly determined by using the ESM. (3) The other five ingredients were indirectly calculated by the QAMS method using RCFs.

Table 6. *RCFs of cinobufotalin to arenobufagin, telocinobufagin, bufotalin, bufalin, resibufogenin.*

Injection volume (μL)	$f_{c/a}$	$f_{c/t}$	$f_{c/bt}$	$f_{c/b}$	$f_{c/r}$
3	1.2235	1.4594	0.7812	1.0195	0.8774
5	1.2162	1.4504	0.7749	1.0286	0.8725
7	1.2123	1.4458	0.7728	1.0206	0.8687
9	1.2154	1.4482	0.7746	1.0415	0.8660
10	1.2136	1.4447	0.7736	1.0364	0.8661
13	1.2144	1.4502	0.7743	1.0306	0.8650
15	1.2139	1.4511	0.7748	1.0682	0.8642
17	1.2141	1.4516	0.7756	1.0379	0.8618
Mean	1.2154	1.4502	0.7752	1.0354	0.8677
RSD%	0.29	0.31	0.33	1.49	0.58

Notes: RCFs: relative correction factors, calculated using the calibration curve; $f_{c/a}$: relative correction factor of cinobufotalin to arenobufagin; $f_{c/t}$: relative correction factor of cinobufotalin to telocinobufagin; $f_{c/bt}$: relative correction factor of cinobufotalin to bufotalin; $f_{c/b}$: relative correction factor of cinobufotalin to bufalin; $f_{c/r}$: relative correction factor of cinobufotalin to resibufogenin.

To validate the differences between ESM and the QAMS methods using RCFs, 41 *Bufonis Venenum* samples were analyzed to determine their active ingredients (Table 7). As demonstrated, there no significant differences between ESM and QAMS methods of all *Bufonis Venenum* samples.

Table 7. *Comparison of the results from the ESM and QAMS (mg mL^{-1}).*

Samlpe No.	Cinobufotalin ESM	Arenobufagin			Telocinobufagin		
		ESM	QAMS	SD	ESM	QAMS	SD
1	0.047	0.055	0.055	0.00	0.009	0.009	0.00
2	0.044	0.013	0.013	0.00	0.007	0.007	0.00
3	0.047	0.061	0.061	0.00	0.011	0.011	0.00
4	0.050	0.049	0.048	0.00	0.009	0.009	0.00
5	0.036	0.025	0.025	0.00	0.009	0.009	0.00
6	0.054	0.055	0.055	0.00	0.007	0.007	0.00
7	0.052	0.067	0.067	0.00	0.009	0.009	0.00
8	0.049	0.046	0.046	0.00	0.009	0.009	0.00
9	0.048	0.061	0.061	0.00	0.011	0.011	0.00
10	0.048	0.037	0.037	0.00	0.009	0.009	0.00
11	0.048	0.036	0.036	0.00	0.009	0.009	0.00
12	0.050	0.063	0.063	0.00	0.010	0.010	0.00
13	0.044	0.035	0.035	0.00	0.008	0.008	0.00
14	0.037	0.027	0.027	0.00	0.009	0.010	0.00
15	0.044	0.043	0.043	0.00	0.018	0.018	0.00
16	0.050	0.066	0.065	0.00	0.010	0.010	0.00
17	0.049	0.036	0.035	0.00	0.009	0.009	0.00
18	0.042	0.007	0.007	0.00	0.005	0.005	0.00
19	0.042	0.053	0.053	0.00	0.018	0.018	0.00
20	0.045	0.035	0.035	0.00	0.009	0.009	0.00
21	0.051	0.044	0.044	0.00	0.007	0.007	0.00
22	0.030	0.016	0.016	0.00	0.014	0.014	0.00
23	0.051	0.031	0.031	0.00	0.011	0.011	0.00
24	0.057	0.015	0.015	0.00	0.008	0.008	0.00
25	0.063	0.023	0.023	0.00	0.010	0.010	0.00
26	0.062	0.017	0.016	0.00	0.009	0.009	0.00
27	0.057	0.019	0.019	0.00	0.007	0.008	0.00

Samlpe No.	Cinobufotalin ESM	Arenobufagin			Telocinobufagin		
		ESM	QAMS	SD	ESM	QAMS	SD
28	0.061	0.020	0.020	0.00	0.011	0.011	0.00
29	0.055	0.014	0.014	0.00	0.007	0.007	0.00
30	0.052	0.014	0.014	0.00	0.007	0.007	0.00
31	0.049	0.062	0.064	0.00	0.011	0.012	0.00
32	0.046	0.014	0.014	0.00	0.007	0.008	0.00
33	0.052	0.060	0.062	0.00	0.009	0.010	0.00
34	0.062	0.008	0.008	0.00	0.009	0.010	0.00
35	0.024	0.027	0.028	0.00	0.017	0.018	0.00
36	0.056	0.018	0.018	0.00	0.006	0.007	0.00
37	0.053	0.019	0.019	0.00	0.010	0.011	0.00
38	0.076	0.011	0.011	0.00	0.008	0.008	0.00
39	0.031	0.095	0.097	0.00	0.019	0.020	0.00
40	0.040	0.050	0.051	0.00	0.012	0.012	0.00
41	0.022	0.003	0.003	0.00	0.006	0.006	0.00

Table 7. *Continued.*

Samlpe No.	Bufotalin			Bufalin			Resibufogenin		
	ESM	QAMS	SD	ESM	QAMS	SD	ESM	QAMS	SD
1	0.024	0.024	0.00	0.021	0.024	0.00	0.022	0.022	0.00
2	0.020	0.020	0.00	0.018	0.020	0.00	0.027	0.026	0.00
3	0.023	0.023	0.00	0.022	0.024	0.00	0.028	0.027	0.00
4	0.027	0.027	0.00	0.024	0.027	0.00	0.022	0.022	0.00
5	0.023	0.023	0.00	0.020	0.020	0.00	0.037	0.037	0.00
6	0.029	0.028	0.00	0.026	0.029	0.00	0.028	0.028	0.00
7	0.029	0.029	0.00	0.025	0.028	0.00	0.023	0.022	0.00
8	0.028	0.028	0.00	0.023	0.026	0.00	0.023	0.023	0.00
9	0.024	0.024	0.00	0.022	0.025	0.00	0.028	0.028	0.00
10	0.032	0.032	0.00	0.024	0.027	0.00	0.036	0.035	0.00
11	0.032	0.032	0.00	0.025	0.027	0.00	0.037	0.036	0.00
12	0.026	0.026	0.00	0.024	0.026	0.00	0.028	0.028	0.00
13	0.029	0.029	0.00	0.022	0.025	0.00	0.034	0.034	0.00
14	0.024	0.024	0.00	0.020	0.023	0.00	0.039	0.038	0.00
15	0.023	0.023	0.00	0.022	0.025	0.00	0.033	0.034	0.00
16	0.023	0.022	0.00	0.022	0.025	0.00	0.032	0.032	0.00
17	0.025	0.025	0.00	0.024	0.027	0.00	0.035	0.035	0.00
18	0.019	0.019	0.00	0.015	0.016	0.00	0.017	0.017	0.00
19	0.020	0.020	0.00	0.018	0.020	0.00	0.022	0.022	0.00
20	0.030	0.030	0.00	0.023	0.026	0.00	0.034	0.034	0.00
21	0.028	0.028	0.00	0.023	0.026	0.00	0.031	0.031	0.00
22	0.021	0.021	0.00	0.017	0.019	0.00	0.022	0.022	0.00
23	0.022	0.022	0.00	0.021	0.023	0.00	0.031	0.031	0.00
24	0.026	0.026	0.00	0.025	0.028	0.00	0.044	0.043	0.00
25	0.034	0.033	0.00	0.025	0.028	0.00	0.027	0.027	0.00
26	0.028	0.028	0.00	0.030	0.033	0.00	0.047	0.047	0.00
27	0.029	0.029	0.00	0.024	0.026	0.00	0.039	0.039	0.00
28	0.031	0.031	0.00	0.028	0.031	0.00	0.038	0.038	0.00
29	0.020	0.020	0.00	0.025	0.028	0.00	0.062	0.062	0.00
30	0.019	0.018	0.00	0.023	0.025	0.00	0.059	0.058	0.00
31	0.024	0.024	0.00	0.022	0.025	0.00	0.004	0.004	0.00
32	0.005	0.005	0.00	0.019	0.022	0.00	0.028	0.028	0.00
33	0.027	0.028	0.00	0.024	0.027	0.00	0.026	0.026	0.00
34	0.037	0.037	0.00	0.029	0.032	0.00	0.056	0.056	0.00
35	0.030	0.030	0.00	0.015	0.017	0.00	0.012	0.012	0.00
36	0.019	0.020	0.00	0.025	0.028	0.00	0.042	0.043	0.00
37	0.030	0.030	0.00	0.023	0.026	0.00	0.029	0.029	0.00
38	0.035	0.035	0.00	0.027	0.031	0.00	0.039	0.039	0.00
39	0.018	0.019	0.00	0.017	0.019	0.00	0.010	0.010	0.00
40	0.021	0.021	0.00	0.018	0.020	0.00	0.022	0.022	0.00
41	0.007	0.007	0.00	0.012	0.013	0.00	0.079	0.080	0.00

3.9. Robustness of QAMS

Since RCF is a key parameter in the application of the QAMS method for the quality control, to understand the influence of chromatographic conditions on RCF values, three types of columns, two instruments, two column temperature (25 and 30°C) and three flow rate (0.5, 0.6 and 0.7 mL min^{-1}) were applied by using the mobile phase

mentioned in Section 2.6. As indicated in Tables 8-10, the RCF values were in good agreement with each other, regardless of the chromatographic conditions employed.

Table 8. *RCFs of different instrument and column of cinobufotalin to arenobufagin, telocinobufagin, bufotalin, bufalin, resibufogenin.*

Instrument	Column	Arenobufagin	Telocinobufagin	Bufotalin	Bufalin	Resibufogenin
	ZORBAX-SB C18	1.0831	1.2602	0.8154	1.0826	0.8629
Agilent	Alltima C18	1.0831	1.2602	0.8154	1.0826	0.8629
	ZORBAX-Bonus-RP	1.0831	1.2602	0.8154	1.0826	0.8629
	ZORBAX-SB C18	1.0886	1.2575	0.8114	1.0847	0.8710
	Alltima C18	1.0886	1.2575	0.8114	1.0847	0.8710
Waters	ZORBAX-Bonus-RP	1.0886	1.2575	0.8114	1.0847	0.8710
	mean	1.0859	1.2589	0.8134	1.0837	0.8670
	RSD%	0.28	0.12	0.27	0.10	0.51

Table 9. *RCFs of different flow rate of cinobufotalin to arenobufagin, telocinobufagin, bufotalin, bufalin, resibufogenin.*

	Flow rate (mL min^{-1})	RCFs				
		$f_{c/a}$	$f_{c/t}$	$f_{c/bt}$	$f_{c/b}$	$f_{c/r}$
	0.5	1.2356	6.1484	1.3759	2.0959	1.4732
	0.6	1.2356	6.1484	1.3759	2.0959	1.4732
25°C	0.7	1.2356	6.1484	1.3759	2.0959	1.4732
	mean	1.2356	6.1484	1.3759	2.0959	1.4732
	RSD (%)	0.00	0.00	0.00	0.00	0.00

Table 10. *RCFs of different column temperature of Cinobufotalin to Arenobufagin, Telocinobufagin, Bufotalin, Bufalin, Resibufogenin.*

Column Temperature (°C)		RCFs				
		$f_{c/a}$	$f_{c/t}$	$f_{c/bt}$	$f_{c/b}$	$f_{c/r}$
	20	1.2356	6.1484	1.3759	2.0959	1.4732
0.7 mL min^{-1}	25	1.2356	6.1484	1.3759	2.0959	1.4732
	mean	1.2356	6.1484	1.3759	2.0959	1.4732
	RSD (%)	0.00	0.00	0.00	0.00	0.00

For better authentication as well as convenience to quality control of *Bufonis Venenum*, the relative retention time (RRT) of the other five components by different columns and instruments was used to identify its chromatographic peak position.

Table 11. *Relative retention time of five components in Bufonis Venenum samples.*

Instrument	Column	Arenobufagin	Telocinobufagin	Bufotalin	Bufalin	Resibufogenin
	ZORBAX-SB C18	0.394	0.585	0.633	0.884	1.028
Agilent	Alltima C18	0.437	0.620	0.692	0.899	1.017
	ZORBAX-Bonus-RP	0.455	0.631	0.694	0.928	1.028
	ZORBAX-SB C18	0.414	0.598	0.648	0.890	1.028
	Alltima C18	0.454	0.635	0.710	0.903	1.017
Waters	ZORBAX-Bonus-RP	0.474	0.648	0.714	0.932	1.027
	mean	0.44	0.62	0.68	0.91	1.02
	RSD/%	3.76	3.80	2.89	2.17	0.53

To improve confidence of authenticity of *Bufonis Venenum*, and due to its convenience for quality control purposes, the relative retention times (RRTs) of the other five components identified using different columns and instruments was used to identify its chromatographic peak position.

Cinobufotalin was explicitly identified and designated as the reference peak in the *Bufonis Venenum* samples (Table 11). The results showed that RRT was stable and could be used to identify the other five compounds referring to cinobufotalin in spite of the differences in columns or HPLC instruments, or those arising from analysis being performed in different laboratories.

4. Conclusions

It is worth noting that bufalin (0.916) and cinobufotalin (0.855) scored higher than other compounds in the PCA (maybe include a mean value, or some value for the other compounds here). It could be inferred that these two compounds were of greater importance in terms of their contribution to the identification of sample origin. Cinobufagin and resibufogenin are detailed as the appropriate control markers to be used for Bufonis Venenum analysis in the Chinese Pharmacopoeia, while bufalin, cinobufagin and resibufogenin are mandated by the Japanese and Korean Pharmacopoeia. In addition, bufalin has been reported to have definite pharmaceutical properties (e.g. anti-cancer, cardiotonic, and analgesic) [7, 9]. Based on our results, bufalin is an important control marker that should be used in the evaluation of Bufonis Venenum quality.

By quantitatively analyzing six major components in Bufonis Venenum, we have shown that, when compared to

HPLC ESM method, the QAMS is a sensitive, simple, and reliable method for multi-component quality control. Moreover, the QAMS method is advantageous in reducing cost and overcoming issues regarding availability of reference substances. When HPLC fingerprint data was combined with similarity analysis, HCA, PCA and QAMS method, the quality of *Bufonis Venenum* could be comprehensively evaluated and more reliably identified. Not only we could distinguish the geographical regions and authentic production areas, but also the origins of *Bufonis Venenum* could be identified. Most importantly, we could distinguish the true from the false through the combination of these methods. Hence, these evaluation methods are promising to be widely applied in the quality control of TCMs.

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