Simultaneous quantification of six alkaloid components from commercial stemonae radix by solid phase extraction-high-performance liquid chromatography coupled with evaporative light scattering detector

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ABSTRACT

Background: Stemonae radix has been applied in traditional Chinese medicine for centuries. Alkaloids are the main active ingredient in stemonae radix, so their composition and concentration levels are directly linked to clinic effects. **Objective:** The objective was to develop an analytical method with multiple markers for quality survey of commercial stemonae radix. **Materials and Methods:** A method for simultaneous determination of six compounds in commercial stemonae radix was performed using solid-phase extraction and high-performance liquid chromatography coupled with evaporative light scattering detector. The separation was carried out on an Agilent TC-C18 column with 0.1% acetonitrile solution of triethylamine aqueous solution and acetonitrile as the mobile phase under gradient elution within 70 min. The hierarchical clustering analysis (HCA) was successfully used to classify the samples in accordance with their chemical constituents. **Results:** Linearity (R^2 > 0.9990), intra- and inter-day precision (relative standard deviations <4%), limit of detection (0.011–0.086 μg/mL), limit of quantification (0.033–0.259 μg/mL) of the six alkaloids were determined, and the recoveries were between 96.6% and 103.7%. The method was successfully applied to analysis 36 batches of commercial stemonae radix. All the samples could be classified into five clusters by HCA. **Conclusion:** This article provides an accurate and simple analytical method for quality survey of commercial stemonae radix. Because of the significant chemical variations, careful selection of *Stemona* sources with obvious antitussive value but devoid of croomine followed by good agricultural practice and good manufacturing practice process is suggested.

Key words: Alkaloid, commercial stemonae radix, quantification, solid-phase extraction and high-performance liquid chromatography coupled with evaporative light scattering detector

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INTRODUCTION

Stemona sessilifolia, *Stemona japonica,* and *Stemona tuberosa* are three authoritative sources of stemonae radix specified in Chinese Pharmacopoeia (CP).[1] They have been applied in traditional Chinese medicine for centuries to manage respiratory diseases, e.g. bronchitis, pertussis, and tuberculosis, and to prevent human and cattle parasites, agricultural pests, and domestic insects.[2,3] Stemona alkaloids are the main active ingredient of stemonae radix; more than 140 alkaloids have been identified so far.[4] In addition to their pharmacological effects, some stemona alkaloids are also proved to show severe physiological toxicities.[5] Among them, croomine showed obvious central respiratory depressant effects, and it might be the major toxic component of stemonae radix.[6]

Significant intraspecific and interspecific chemical diversities were found among the three authoritative

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Stemona species,[7] which made the authentication and quality control difficult. Total alkaloid contents were chosen as a quality marker in CP, but it has limited value as an indicator because the antitussive activity varied with the change of alkaloid composition, and some samples contained high concentrations of toxic alkaloids (such as croomine can lead to respiratory depression). Since most of the stemona alkaloids are not sensitive under direct ultraviolet detection due to the absence of conjugated functional groups, high-performance liquid chromatography coupled with evaporative light scattering detector (HPLC-ELSD) and mass spectrometry (MS) detections are commonly used.[8] For examples, an online HPLC-ESI/MS analysis method was performed to monitor the stemona alkaloids from three pharmacopoeia species,[9] and HPLC-ELSD was developed to simultaneously quantify six major bioactive alkaloids in stemonae radix.[10] Though analytical methods have been established for the qualitative detection or quantitative determination of alkaloids in stemonae radix. All these studies are performed on single collected species of *Stemona*. The fact is that the herbal material of stemonae radix used in clinics is mainly from commercial sources which might contain a mixture of different species. The alkaloid composition and concentration levels, especially the toxiferous alkaloid, are directly linked to the clinic effects. Thus, a detailed survey of commercial stemonae radix is warranted.

The objective of the present study was to develop an analytical method with multiple markers for quality survey of commercial stemonae radix. For this purpose, a hyphened method of solid phase extraction and HPLC-ELSD (SPE-HPLC-ELSD) was established to explore the chemical profiles of commercial stemonae radix purchased from different herbal shops. Six alkaloids, including croomine (1), stemoninine (2), tuberostemonine (3), neotuberostemonine (4), bisdehydrostemonine (5), tuberostemonine D (6), and tuberostemonine K (7) were selected as the chemical markers for their quality survey. The validated method was applied to compare the chemical profiles of 36 batches of commercial stemonae radix. Finally, hierarchical clustering analysis (HCA) was used for the classification of these samples. The proposed method proved to be accurate and simple and could be used for the quality survey of commercial stemonae radix.

EXPERIMENTAL

Chemicals, reagents, and materials

Chemical standards of croomine (1), stemoninine (2), tuberostemonine (3), neotuberostemonine (4), bisdehydrostemoninne (5), tuberostemonine D (6), and tuberostemonine K (7) were isolated from *Stemona* plants in our laboratory, and their structures were determined by nuclear magnetic resonance and single-crystal X-ray diffraction [Figure 1]. The identities of these chemicals were confirmed by comparisons of their spectral data with those had been published.[7,10-14] The purity of each reference compound was determined to be over 98% by normalization of the peak area detected by HPLC-ELSD.

Acetonitrile was HPLC-grade from Aladdin Chemistry Inc. (Shanghai, China). Deionized water was obtained using a Milli-Q water purification system (MilliPore, MA, USA). Other reagent solutions were of analytical grade (Shanghai Chemical Co., Shanghai, China).

The commercial stemonae radix was purchased from different herbal shops of China in 2012. Their botanical origins were identified by Dr. Ying Zhang. Voucher specimens were deposited at College of Pharmacy, Jinan University. All the samples were dried at 50°C.

Apparatus and chromatographic conditions

Quantitative analysis was performed on an Agilent 1200 liquid chromatography system (Santa Clara, USA), equipped with ELSD, a quaternary solvent delivery system, a column temperature controller, and an autosampler. Chromatographic data were recorded and processed with Allchrom Plus Client/Server software. An Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) and C18 guard column (5 μm, 4.6 mm × 12.5 mm) were used and maintained at 25°C. The mobile phase was 0.1% triethylamine aqueous solution (A) and acetonitrile (B) with a gradient program as follows: 0–10 min, linear gradient 20–30% B; 10–25 min, linear gradient 30–40% B; 25–35 min, isocratic elution 40% B; 35–45 min, linear gradient 40–50% B; 45–55 min, linear gradient 50–55% B; 55–70 min, linear gradient 55–80% B and the post run (10 min) at a flow rate of 1.0 mL/min. The temperature for the ELSD drift tube was set at 97°C, and the nitrogen flow was 3.0 standard liter/min. The injection volume was 30 μL. Representative chromatograms for the standard analytes and the samples are shown in Figure 2.

Preparation of sample solutions

The dried powder of stemonae radix samples (1.0 g, 80 meshes) was accurately weighed and added into a conical flask (100 mL), then 50 mL methanol was added. After soaking for 30 min, the sample was refluxed for 30 min. The total extract was filtered, and 25 mL filtrate was concentrated under reduced pressure to afford a residue, which was suspended in 2 mL of distilled water. The suspension was transferred to a C18 SPE column, which was eluted with 5 mL distilled water followed by 5 mL methanol. The methanol eluate was transferred to a

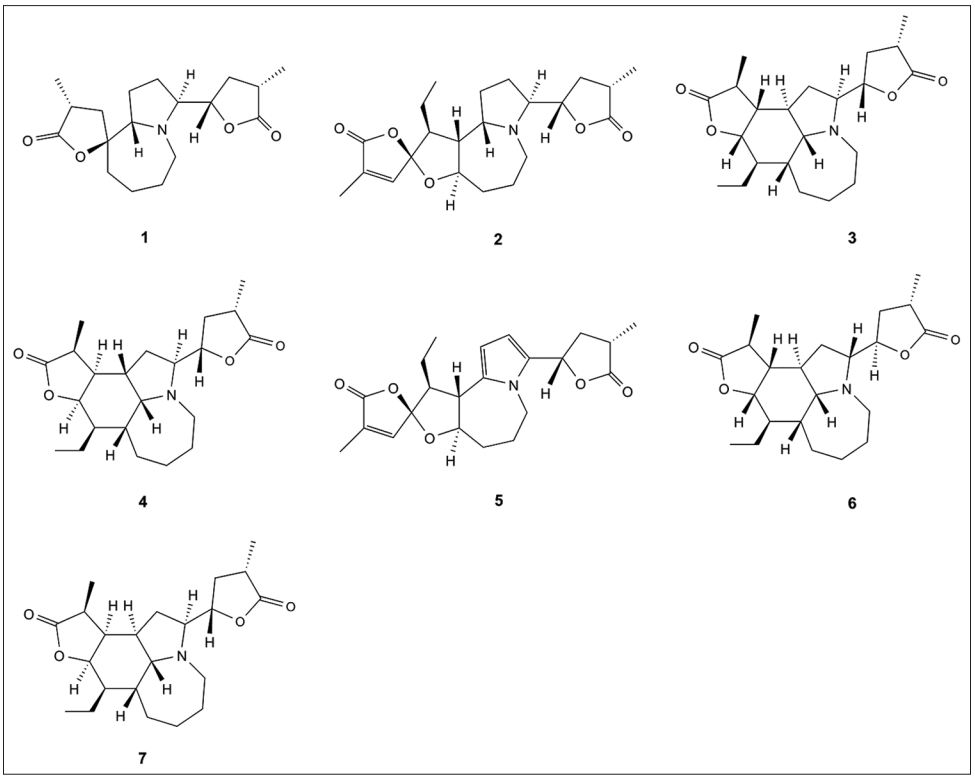

Figure 1: Chemical structures of the identified compounds of stemonae radix: Croomine (1), stemoninine (2), tuberostemonine (3), neotuberostemonine (4), bisdehydrostemonine (5), tuberostemonine D (6), tuberostemonine K (7)

5 mL volumetric flask, and then diluted with methanol to the mark. An aliquot of the methanol solution was filtered with a 0.45 μm membrane filter before HPLC injection.

Validation of the high-performance liquid chromatography method

Calibration curves, limits of detection and quantification

The mixed standard solution containing 0.543 mg/mL of croomine, 0.721 mg/mL of stemoninine, 0.611 mg/mL of tuberostemonine, 0.563 mg/mL of neotuberostemonine, 0.910 mg/mL of bisdehydrostemonine and 0.881 mg/mL of tuberostemonine D were prepared by dissolving each standard in a volumetric flask with 5 mL of methanol. A series of working solutions with gradient concentration was obtained by diluting the mixed standard solution. The calibration curve for each compound was established by plotting the logarithm of the peak area (y) versus the logarithm of the injection concentration amount (x) of each analyte. The limit of detection (LOD) and limit of quantification (LOQ) for six analytes were estimated at signal-to-noise ratios of 3 and 10, respectively, by injecting a series of dilute solutions with known concentrations.

Precision, repeatability, and accuracy

Intra- and inter-day variations and repeatability were chosen to determine the precision of the developed assay. For intra-day variability test, the mixed standard solutions were analyzed for six replicates within 1 day while for inter-day variability test, the solutions were examined in duplicates for consecutive 3 days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD). The repeatability of the method was evaluated by analyzing the contents of compounds 1–6 in six independently prepared samples of stemonae radix.

A recovery test was used to evaluate the accuracy of the method. The recovery test was carried out by standard addition method. Six alkaloids in a mixed standard solution were spiked into the samples (1.0 g), and then extracted, processed, and quantified in accordance with the established procedures.

Data analysis and quality evaluation

Hierarchical clustering analysis is a multivariate analysis technique that is used to classify samples into groups.[15] In this study, the HCA of 36 batches of commercial samples was performed using Statistical Package for the Social Sciences (SPSS) software (SPSS for Windows 13.0, SPSS Inc., Chicago, IL, USA). The Ward's method as the amalgamation rule and the Squared Euclidean Distance

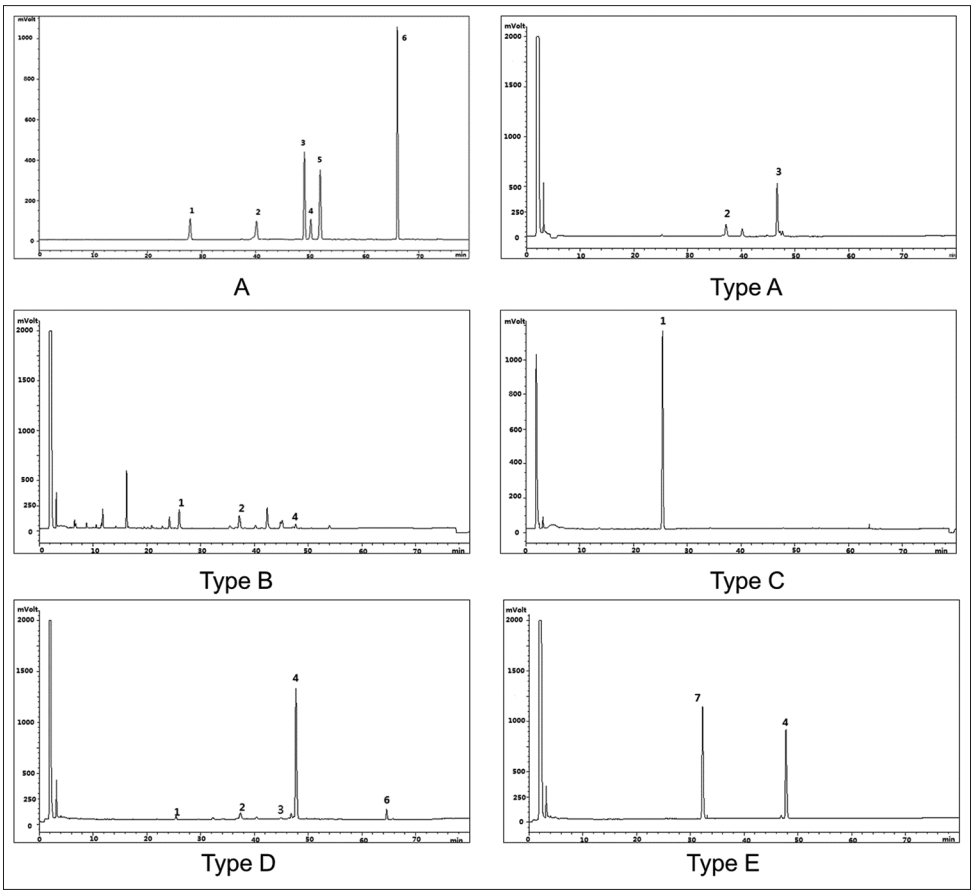

Figure 2: The typical chromatogram of the standard mixtures (A); the typical chromatogram of representative samples. (Type A: S10; Type B: S2; Type C: S24; Type D: S36; Type E: S35). 1: Croomine, 2: Stemoninine, 3: Tuberostemonine, 4: Neotuberostemonine, 5: Bisdehydrostemonine, 6: Tuberostemonine D, 7: Tuberostemonine K

as metric were used to establish clusters. The results of this hierarchical clustering process were represented in a dendrogram.

RESULTS AND DISCUSSION

Optimization of sample extraction methods

Reflux and ultrasonic extractions were compared, and the former obtained better extraction yield. The extraction efficiency of 95% ethanol and methanol was evaluated, and it was found that methanol was the most suitable solvent for extraction. Comparison of the extraction time 30, 60, and 90 min under reflux condition in methanol showed that the complete extraction of the compounds could be achieved within 30 min. Therefore, reflux extraction under reflux condition for 30 min in methanol was chosen as the optimal extraction method for following experiments.

Two purification methods for enrichment of total alkaloids, SPE and liquid-liquid extraction (LLE) were compared, and it was observed that the recovery of SPE is much higher than that of LLE. The relevant parameters of SPE, such as sample amount, pH value of sample and elution volume, were optimized in order to achieve satisfactory purification efficiency. Finally, the SPE purification procedure was summarized as following: (i) The total extract was suspended in 2 mL water, then the suspension was transferred to a C18 SPE column; (ii) the SPE column was eluted with 5 mL water to remove the water-soluble substances, and then eluted with 5 mL methanol to get the total alkaloids fraction; and (iii) the methanol elute was transferred to a 5 mL volumetric flask, and then diluted with methanol to the mark.

High-performance liquid chromatography method validation

The proposed HPLC-ELSD method for quantitation analysis was validated to determine the linearity, LOD, LOQ, intra-day and inter-day precisions, repeatability, and accuracy. As shown in Table 1, all calibration curves showed good linearity ($R^2 > 0.9990$) within the test ranges, and the overall LODs and LOQs were in the ranges of 0.011–0.086 μg/mL and 0.033–0.259 μg/mL, respectively. The RSD values of intra- and inter-day variations, and

repeatability of the six analytes were all <3.4%, and the overall recoveries lay between 96.6% and 103.7% with RSD < 4% [Table 2]. All the results mentioned above indicated that the established method was accurate enough for the determination of the six chemical markers in the samples of stemonae radix.

Quantitative analysis of different batches of stemonae radix

The developed HPLC-ELSD method was applied to simultaneous determination of the chemical markers including croomine (1), stemoninine (2), tuberostemonine (3), neotuberostemonine (4), bisdehydrostemoninne (5), and tuberostemonine D (6) in 36 batches of commercial stemonae radix. The results are shown in Table 3 as the mean values of the three replicate injections. It is clear that there were remarkable differences among the contents of alkaloids analyzed in different samples. For example, the content of croomine reaches up to 30.93 mg/g in sample S24, and there is almost no other alkaloid in this sample. Bisdehydrostemonine exists only in the three samples (S9, S13, S16), and the content is very low. Then stemoninine, tuberostemonine, and neotuberostemonine are more widely distributed, and the contents varied among different batches.

Hierarchical clustering analysis

Significant intraspecific and interspecific chemical diversities are found among the three pharmacopoeial species. In order to evaluate the variation of commercial stemonae radix, HCA was performed based on all of the components characteristics from HPLC profiles of 36 batches of samples. The results of HCA are shown in Figure 3. All the samples could be classified into five clusters: Type A with the major component being tuberostemonine (3) and stemoninine (2) (S10, S25, S27, S30, S11, S31, S26, S8, S9, S16, S32, S13, S14, S17, S15, S07, S34, S22), type B with the major component being mixing alkaloids (S02, S12, S06, S28, S01), type C with the major component being croomine (1) (S24), type D with the major component being neotuberostemonine (4) (S04, S19, S21, S23, S36, S20, S29, S03), type E with the major component being tuberostemonine K (7) and neotuberostemonine (4) (S18, S35, S05, S33), as shown in Figure 2. Though compounds 5 and 6 were quantified, they were not the major components in these samples.

CONCLUDING REMARKS

This study provided an SPE-HPLC-ELSD method for simultaneous quantification of six alkaloids, namely, croomine, stemoninine, tuberostemonine, neotuberostemonine, bisdehydrostemonine and tuberostemonine D in commercial stemonae radix. Based on the validation results of good accuracy, repeatability, and precision, the proposed method could be adopted as a prerequisite for quality control of stemonae radix.

In addition, HCA analysis was successfully used for differentiation and evaluation of samples qualities. Significant variations in the contents of the six alkaloids were found in 36 samples of stemonae radix from different commercial sources. It has been reported that significant

Table 1: Linear equation, LOQs and LODs of six investigated compounds

Peak number	Compound	Linear equation[a]	R^2	Linear range (μg/mL)	LOQ[b] (μg/mL)	LOD[c] (μg/mL)
1	Croomine	y=1.3878x+5.1565	0.9992	1.76-35.2	0.219	0.073
2	Stemoninine	y=1.3992x+4.3218	0.9994	1.82-36.4	0.259	0.086
3	Tuberostemonine	y=1.4293x+4.8628	0.9995	1.13-22.5	0.109	0.036
4	Neotuberostemonine	y=1.3013x+4.4997	0.9991	1.22-24.4	0.203	0.067
5	Bisdehydrostemoninne	y=1.3907x+4.6437	0.9995	1.44-28.8	0.218	0.072
6	Tuberostemonine D	y=1.4161x+4.9246	0.9993	1.09-21.7	0.033	0.011

[a]y and x are, respectively, the peak areas and concentrations (μg/mL) of the analytes. [b]LOD: Limit of detection (S/N=3), [c]LOQ: Limit of quantification (S/N=10)

Table 2: Precision, repeatability, and accuracy of six investigated compounds

Peak number	Compound	Precision (RSD, %)[a]		Repeatability (%)[a] (RSD, *n*=6)	Recovery (%, *n*=6)	
		Intra-day (*n*=6)	Inter-day (*n*=18)		Mean	RSD (%)
1	Croomine	2.78	2.69	0.75	98.44	2.51
2	Stemoninine	2.07	2.41	0.87	97.72	4.16
3	Tuberostemonine	3.00	2.23	1.08	103.69	0.83
4	Neotuberostemonine	2.75	2.21	1.32	101.10	2.18
5	Bisdehydrostemoninne	2.61	2.60	0.65	96.63	2.24
6	Tuberostemonine D	2.91	3.32	0.98	97.70	3.53

[a]RSD (%)=(SD of amount detected/mean of the amount detected)×100. RSD: Relative standard deviation, SD: Standard deviation

Table 3: The contents (mg/g) of six alkaloid components in 36 samples of stemonae radix

Sample	Code[a]	Sources	Content (mg/g)					
			1[b]	2	3	4	5	6
1	Sj	Guangxi	12.46	30.86	-[c]	3.80	-	5.76
2	Ss+St	Guangxi	27.67	21.78	-	12.95	-	-
3	St	Yunnan	-	-	-	92.75	-	-
4	Ss+St	Hubei	-	-	-	20.59	-	-
5	Ss+St	Guangxi	-	-	4.76	21.96	-	-
6	Sj+St	Anhui	5.98	12.80	5.85	6.96	-	-
7	Ss	Anhui	-	22.01	-	2.00	-	-
8	St	Guangxi	1.61	5.07	2.96	2.65	-	-
9	St	Guangxi	5.97	-	-	7.15	3.07	-
10	St	Guangxi	-	18.29	14.75	8.51	-	-
11	Sj+Ss	Yunnan	-	18.03	5.31	11.84	-	-
12	Sj	Anhui	7.43	19.92	-	9.70	-	-
13	St	Guangxi	4.23	15.72	-	29.44	4.33	1.51
14	Ss+St	Guangxi	-	54.40	-	-	-	-
15	St	Guangxi	9.61	62.32	-	-	-	-
16	Sj+St	Guangxi	4.65	15.40	-	-	3.16	-
17	St	Guangxi	3.08	63.15	-	-	-	-
18	St	Jiangsu	-	-	-	50.45	-	-
19	St	Juangsu	-	-	-	50.89	-	-
20	St	Anhui	-	-	-	125.34	-	-
21	Sj+St	Hubei	-	-	-	49.77	-	0.64
22	Ss	Guangxi	-	35.76	-	15.15	-	-
23	St	Guangxi	1.31	8.59	-	56.58	-	2.03
24	Sj+St	Guangxi	30.93	-	-	-	-	-
25	Sj	Sichuan	6.77	-	15.41	7.51	-	-
26	Ss+St	Yunnan	-	14.54	-	19.21	-	1.37
27	St	Guangxi	-	13.44	16.54	8.17	-	-
28	Sj+Ss	Hubei	8.67	24.78	11.04	13.91	-	-
29	Ss	Anhui	-	-	-	134.73	-	2.24
30	St	Guangxi	-	21.12	9.13	1.06	-	-
31	Sj+Ss	Anhui	-	11.55	3.92	9.59	-	-
32	Sj	Guangxi	6.70	28.41	-	6.39	-	-
33	Ss+St	Guangxi	-	-	13.18	16.27	-	-
34	Ss+St	Hubei	-	40.64	3.93	-	-	-
35	Ss+St	Jiangxi	-	-	2.57	55.68	-	-
36	Ss	Hubei	4.80	11.33	3.26	76.69	-	0.64

[a]Ss: *Stemona sessilifolia*; Sj: *Stemona japonica*; St: *Stemona tuberosa*. [b]The analysts are the same as in Figure 2 and Table 1; [c]Undetected

intraspecific and interspecific chemical diversities existed in *Stemona* genus. Our results showed the first time that commercial stemonae radix exhibited even larger variations. Variations of single *Stemona* species might be due to genetic parameters and environmental factors, such as differences in age, seasons of collection, and geographic distributions. For commercial samples, chemical variation was further increased by the mixed species. Among the 36 samples, 15 batches were a mixture of two authoritative sources of stemonae radix specified in CP.

Cough is one of the most common symptom for which medical advice is sought.[16] Antitussives are among the most widely used drugs in the world. stemonae radix is a good candidate for antitussive therapy. Bioassays in guinea pigs have documented its antitussive properties.[2]

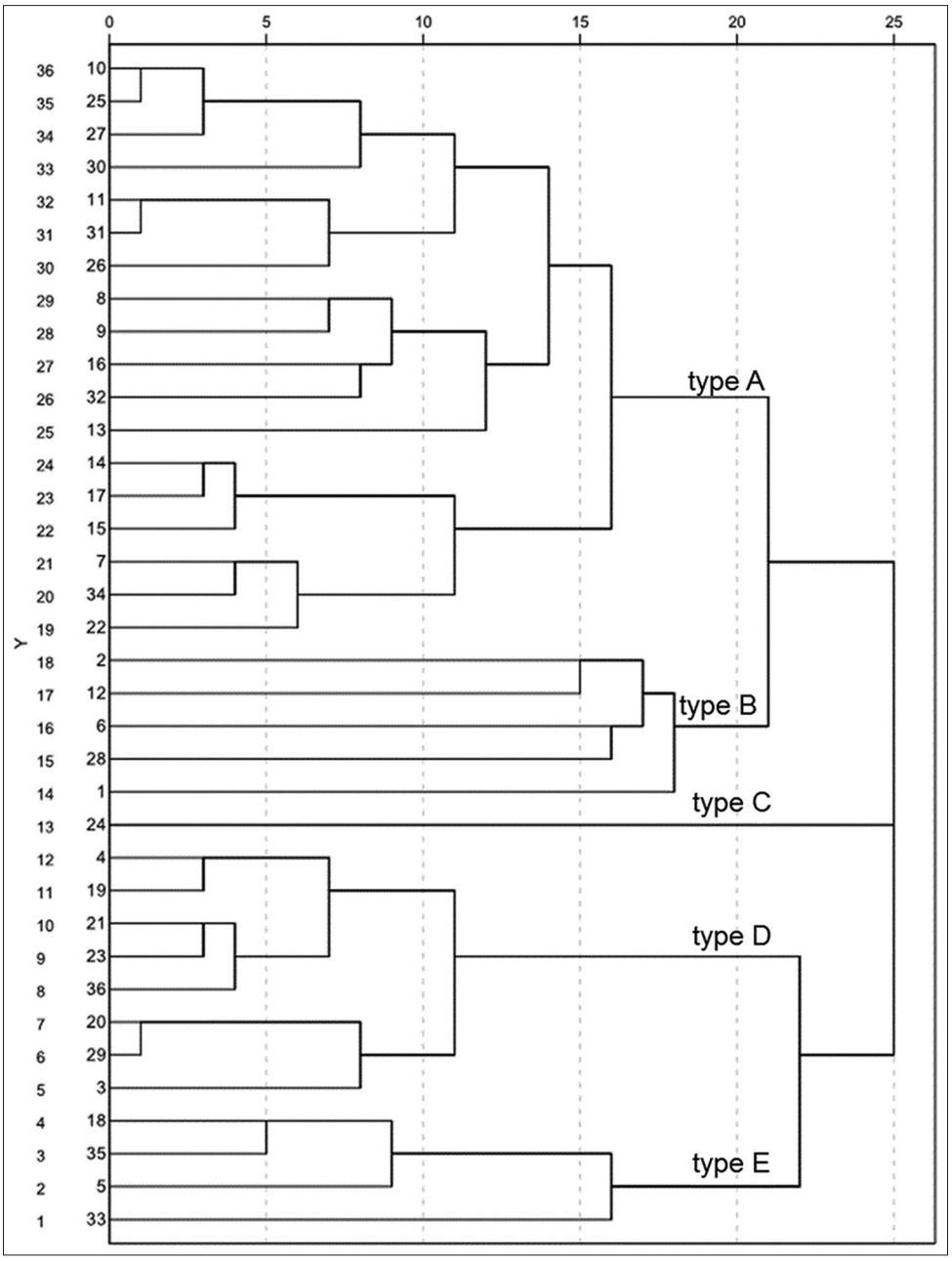

Figure 3: Dendrograms of hierarchical cluster analysis for the 36 tested samples of stemonae radix, dendrogram resulting from the six investigated compounds peaks' area derived from high-performance liquid chromatography profiles of the tested samples

However, the alkaloid composition changes batch by batch and some batches even contain toxic alkaloid croomine.[7] Thus, it is not possible to achieve the same effect when using the same amount of herbal material from different herbal shops. It would be strategically possible to select samples high in stemona alkaloids with obvious antitussive value but low or devoid of croomine, especially for good agricultural practice farms and good manufacturing practice manufacturers. The method developed in this study can help monitor the contents of stemona alkaloids for quality assurance purposes.

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