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DEVELOPMENT OF A FINGERPRINT METHOD FOR ANIMAL HORN CLASSIFICATION BY LIQUID CHROMATOGRAPHY COUPLED WITH HIERARCHICAL CLUSTERING ANALYSIS

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□ *A validated liquid chromatography (LC) method coupled with hierarchical clustering analysis (HCA) has been established for investigating the fingerprint chromatograms of ten kinds of animal horns. The method showed good performances in terms of precision, repeatability, linearity, accuracy, and stability. The results showed that the LC fingerprint method coupled with HCA could be used to classify animal horns, which could make it simple and feasible to search substitution of precious horns.*

Keywords animal horns, classification, fingerprint chromatogram, hierarchical clustering analysis, liquid chromatography, quantification

INTRODUCTION

Animal horns are pointed projections of the skin on the heads of animals, consisting of a covering of horn sheath surrounding by a core of living bone. Traditional Chinese medicine (TCM) derived from animal horns is an important part of Chinese medicine. The horn-derived TCMs such as *Cornu Rhinoceri Asiatici* (rhinoceros horn, RH), *Cornu Bubali* (water buffalo horn, WBH), *Cornu Saigae Tataricae* (Saiga antelope horn, AH), and *Cornu Bovis Grunniens* (yak horn,YH) have been used for over 1,000 years. According to the record of Chinese ancient materia medica, such as *Shennong Bencao Jing* (about 200 B.C.–200 A.D.), *Ming Yi Bie Lu* (about 220 A.D.), and *Bencao Gangmu* (about 1590 A.D.), animal horns

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could be used for dispelling heat, counteracting toxins, and relieving convulsions.[1,2]

Since the 1970s, rhinoceros have been listed as endangered by the Convention on International Trade in Endangered Species (CITES) of Wild Animals and Plants and, therefore, RH was forbidden to be used in China.[3] In order to reduce the dependence on RH in Chinese medicine, researchers have started to look for other horns that could substitute for RH. Different animal horns have been investigated to determine their inorganic elements, amino acids constituents, and pharmacological properties in recent decades.[4] As a result, WBH were shown to be similar to RH in all of the aspects previously described.[5,6]

The requirement to provide classification of animal horns and to find suitable substitutions of precious horn-derived TCM (such as RH and AH) reasonably and conveniently necessitates that a new method of analysis be established. Currently, fingerprint analysis has been internationally accepted as an efficient technique to identify herbal medicines.[7–10] Compared with the previous methods on classifying animal horns, such as inorganic elements analysis, amino acids analysis, and pharmacological properties research, liquid chromatography (LC) fingerprint analysis can be more simple and efficient.

In the present study, LC fingerprints of animal horns were initially established and used to classify animal horns. Hierarchical clustering analysis (HCA) was used to analyze the fingerprint data of animal horns and to generate a visual plot on classification of animal horns. Additionally, an active horn peptide (HP) was also quantified. According to our previous study, HP was first purified from WBH, which possesses antioxidant activity and might contribute to the efficacy of WBH.[11]

EXPERIMENTAL

Chemical, Standards, and Material

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Double-distilled water was prepared in our laboratory (Millipore, Bedford, MA, USA). Phosphoric acid was purchased from Shanghai Chemical (Shanghai, China). Chemical standard of horn peptide (HP, Ala-Ala-Asp-Asn-Ala-Asn-Glu-Leu-Phe-Pro-Pro-Asn) was purified from WBH in our previous work and the peptide sequence was confirmed by matrix assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS).[11]

Cornu Rhinoceri Asiatici (RH-1), *Cornu Rhinoceri Africani* (RH-2), and *Cornu Pantholopsis Hodgsoni* (Tibetan antelope horn, TAH) was made

available from the State Forestry Administration of China (2006, No. 1093). *Cornu Bubali* (WBH) and *Cornu Caprae Hircus* (goat horn, GH) were collected from Wharf town of Huaian city, Jiangsu Province, China. Based on the different fur color, yaks could be divided into white yak, black yak, and motley yak. Three kinds of *Cornu Bovis Grunniens*, white YH (YH-1), black YH (YH-2) and motley YH (YH-3), from different color yaks were collected from Tibet, China. Pulvis *Cornus Bubali* Concentratus (WBHC) and *Cornu Saigae Tataricae* (AH) were purchased from Jiangsu Medicine Company, China. The sample number and abbreviation of each horn is shown in Table 1. The voucher specimens were deposited at the Herbarium in Nanjing University of Chinese Medicine, China. Horns were authenticated by Prof. Jin-ao Duan and were then pulverized into fine powder. WBHC was prepared from WBH and met the standard of Chinese Pharmacopoeia 2010 Edition.[12]

Instrumentation and Chromatographic Conditions

The LC analysis was performed with a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA), consisting of a quaternary pump solvent management system, an on-line degasser, and an autosampler. The raw data were detected with Waters 2998 photodiode array detector, acquired, and processed with Empower Software. A WondaSil C_{18} reversed-phase column (5 μm, 250 × 4.6 mm) with an Alltima-C_{18} guard column (5 μm, 10 × 4.6 mm) of the same stationary phase was used. The column temperature was set at 25°C. The mobile phase was composed of solvents A (0.1% aqueous phosphoric acid) and B (methanol) with a linear gradient elution: 0–25 min, 1% B; 25–70 min, 1–40% B; 70–90 min, 40% B. The flow rate was applied as follow: 0–22.5 min, 0.5 mL/min; 22.5–25 min, 0.5–1 mL/min; 25–90 min, 1 mL/min. Re-equilibration duration was 15 min between individual runs. A detection wavelength of the schematic diagram of 254 nm was selected.

TABLE 1 Collection of Animal Horns and the Quantification of HP Standard

Sample	Sample Number	Sample Abbreviation	Content of HP (mg/kg)
Cornu Rhinoceri Asiatici	S1	RH-1	120.555 ± 5.358
Cornu Rhinoceri Africani	S2	RH-2	138.843 ± 3.545
Cornu Pantholopsis Hodgsoni	S3	TAH	123.799 ± 5.324
Cornu Saigae Tataricae	S4	AH	86.738 ± 1.698
Cornu Bovis Grunniens (White yak)	S5	YH-1	21.541 ± 0.357
Cornu Bovis Grunniens (Black yak)	S6	YH-2	24.350 ± 0.554
Cornu Bovis Grunniens (Motley yak)	S7	YH-3	15.310 ± 1.295
Cornu Caprae Hircus	S8	GH	20.472 ± 0.823
Cornu Bubali	S9	WBH	17.343 ± 1.190
Pulvis *Cornus Bubali* Concentratus	S10	WBHC	95.441 ± 3.070

Sample Preparation

Accurately weighed powder, 2.0 g of each sample, was ultrasonically extracted with 20 mL water for 60 min in a conical flask. The extracted sample was stirred and centrifuged at 1,200 g for 15 min, and then an aliquot (1.5 mL) was taken from the supernatant. All sample solutions were filtered through a 0.22 μm nylon syringe and 20 μL of the filtrate were introduced into the LC system.

Precision, Repeatability, and Stability Test

To evaluate validation of the method, Sample 1 (RH-1) was extracted. The precision test was determined by replicate injection of the same sample solution for five times. The repeatability test was analyzed by injecting five independently prepared samples. The stability test was determined by five injections with one sample solution during 3 days. The RSD of relative retention times (RRT) and relative peak areas (RPA) of each test were calculated.

LC Quantitation of HP: Calibration, Linearity, and Recovery

HP standard was accurately weighed, transferred to volumetric flasks, and dissolved in 15% methanol to make individual stock solutions of 1000 mg/L. Working standards at the concentration of the calibration range were prepared by dilution of the stock solution with 15% methanol. The concentrations of dilution were: 1, 2, 4, 8, 20, 40, and 100 mg/L. Three injections were performed for each dilution. The standard curve was obtained by peak area/corresponding concentration of the injected standard solutions, and the concentrations of HP in the samples were calculated based on the calibration curve.

The stock solution of HP was added to untreated Sample 1 to yield final concentration. The samples were allowed to settle for 30 min prior to extraction. They were later processed according to the aforementioned sample preparation procedure. The experiment was repeated five times.

The limits of detection (LOD) and quantification (LOQ) were evaluated on the basis of signal-to-noise ratios of 3 and 10, respectively.

Data Analysis

The chromatographic profiles of all extracts were performed by professional software Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A), which was recommended by the State

Food and Drug Administration (SFDA) of China for evaluating similarities of chromatographic profiles of TCM. Eleven common peaks in the chromatograms were selected and the peak of HP at retention time 52.196 min was used as a reference. RRT and RPA of each common peak to reference were calculated in the chromatograms.

The hierarchical clustering analysis (HCA) of ten samples was performed based on the variation patterns of eleven chemical constituents of each sample using SPSS software (SPSS for Windows 16.0, SPSS Inc., USA). Ward's method was applied, and Squared Euclidean distance was selected as a measurement.

RESULTS AND DISCUSSION

Optimization of Extraction Conditions

In order to obtain sufficient information about the chemical constituents of samples, extraction conditions were optimized. It has been reported that horn aqueous extraction shows good bioactivities.[1,2,11] Thus, water was chosen as the extraction solvent. Then, different extraction methods were compared, including heat-reflux and ultrasonic extraction for 0.5, 1, and 2 hr, respectively. It was proven that the efficiency of ultrasonic extraction was higher than heat-reflux extraction. Furthermore, it was found that ultrasonic extraction for 1 hr was sufficient to extract the analytes. The results indicated that the optimal extraction procedure was as follows. Each sample was extracted by ultrasonic extraction for 1 hr using aqueous solvent.

Optimization of LC Conditions

In order to obtain an acceptable chromatographic profile, column, detection wavelength, mobile phase, flow rate and gradient were optimized. Three brands of analytical columns, WondaSil C_{18} reversed-phase column (5 μm, 250 × 4.6 mm), Apollo C_{18} column (5 μm, 250 mm × 4.6 mm), and Waters SunFire C_{18} (5 μm, 250 mm × 4.6 mm) were compared. The results showed that the WondaSil C_{18} column was more suitable for the samples in this study. The spectra of all the main peaks were investigated by a photodiode array detector; in a comparison with the chromatographic profile in 210 nm and 280 nm, 254 nm was selected as the detection wavelength to obtain a sufficient number of detectable peaks in the chromatograms. As for the mobile phase, methanol-water, acetonitrile-water, methanol-0.1% aqueous acetic acid, and methanol-0.1% aqueous phosphoric acid were investigated and the chromatogram with the best separation was acquired

when methanol-0.1% aqueous phosphoric acid was selected as the mobile phase. In 0–25 min, the resolution of the characteristic peaks at 0.5 mL/min flow rate was better than at 1 mL/min. Therefore, the optimum flow rate was chosen as 0.5 mL/min in 0–22.5 min, 0.5–1 mL/min in 22.5–25 min, and 1 mL/min in 25–90 min. In the process of gradient optimization, the gradient program was chosen as 0–25 min, 1% B; 25–70 min, 1–40% B; 70–90 min, 40% B.

Validation of the Method

Precision Test

The precision of injection was determined by replicate injections of the same solution (Sample 1) for five times; the results indicated that the RSDs of RRT and RPA were lower than 0.55 and 2.08%, respectively.

Repeatability Test

The repeatability test was assessed by analyzing five independent extracts prepared samples (Sample 1) using the same method. The results indicated that the RSDs of RRT and RPA were lower than 0.78 and 2.63%, respectively.

Sample Stability Test

The sample stability test was validated with one prepared extract of the sample during 3 days. During this period, the solution was stored at room temperature. The results indicated that the RSDs of RRT and RPA were lower than 0.43 and 2.91%, respectively. The results indicated that the extract of sample remained stable for at least 3 days.

LC Fingerprint Analysis

Ten samples were extracted and analyzed in present study. In fingerprint analysis, 11 common peaks shown in the fingerprint chromatogram were assigned as characteristic peaks. The fingerprint of Sample 1 was selected as reference for automatic matching. As shown in Figure 1, the chromatographic fingerprints of ten samples were compared.

Calibration, Linearity, and Recovery

The calibration curve for HP was performed with seven different concentrations by plotting the peak area versus concentration. Linear regression analysis for HP was performed by the external standard method. Calibration plot for HP showed good linearity within the test range investigated. The equation was calculated as follow, $y = 31{,}056\ \ x - 3{,}896.5$

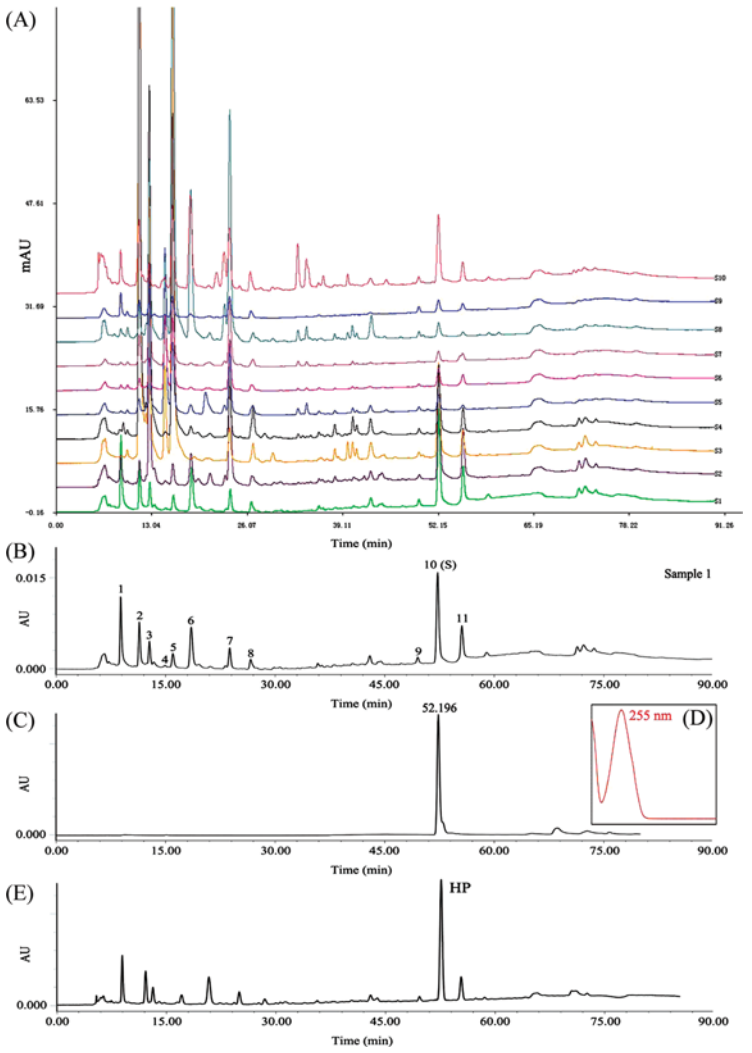

FIGURE 1 The chromatographic fingerprints obtained from animal horns: (A) The chromatographic fingerprints of ten different animal horns; (B) The chromatographic fingerprints obtained from sample 1, and eleven common peaks were signed on the chromatogram; (C) Chromatogram of the standard horn peptide; (D) Ultraviolet spectrum of the standard horn peptide; and(E) Chromatogram of the spiked sample 1. (Color figure available online.)

$r^2 = 1.0000$. Recovery was tested to evaluate the method accuracy. Sample 1 was added with standard HP solution, then extracted, processed, and quantified utilizing the same method, and three replicate analyses were performed. Average recovery of HP was 100.71%. The LOD and LOQ obtained for HP were 0.1 μg/mL and 0.03 μg/mL.

Quantification of HP

It was found that the active horn peptide HP could be detected in ten samples and was identified by comparison of their retention times (RT) and UV absorption spectra with standard HP (shown in Figure 1). As shown in Table 1, the content of HP in ten samples was different, which implied the efficacy of each sample was different.

Hierarchical Cluster Analysis of the Samples

Ten samples were derived from nine different kinds of animals. The chromatogram of ten samples contained eleven common constituents. According to the contents and bioactivity properties, Peak 10 (HP) was chosen as reference peak for calculation of RRT and RPA. The values of RPA for the 10 samples is shown in Table 2. In order to classify the samples, HCA was used to compare the chromatograms of 10 samples on the basis of RPA values by SPSS software. As shown in Figure 2, when using eleven common peaks as the clustering variable, the horns from different animals

TABLE 2 Relative Retention Times and Relative Areas of the Common Peaks

	Peak										
Sample	1	2	3	4	5	6	7	8	9	10(S)	11
					RRT						
S1	0.170	0.218	0.245	0.286	0.306	0.354	0.455	0.510	0.948	1.000	1.063
S2	0.168	0.217	0.243	0.284	0.304	0.352	0.454	0.510	0.949	1.000	1.063
S3	0.171	0.217	0.243	0.284	0.304	0.361	0.453	0.515	0.948	1.000	1.064
S4	0.167	0.217	0.243	0.285	0.304	0.352	0.454	0.515	0.949	1.000	1.064
S5	0.169	0.217	0.243	0.284	0.304	0.351	0.454	0.514	0.949	1.000	1.063
S6	0.169	0.218	0.243	0.284	0.304	0.351	0.454	0.511	0.949	1.000	1.063
S7	0.169	0.217	0.243	0.285	0.305	0.353	0.454	0.515	0.948	1.000	1.064
S8	0.168	0.217	0.243	0.284	0.304	0.352	0.454	0.515	0.949	1.000	1.063
S9	0.168	0.217	0.243	0.284	0.304	0.351	0.453	0.509	0.949	1.000	1.063
S10	0.170	0.219	0.245	0.287	0.306	0.355	0.443	0.499	0.950	1.000	1.064
RSD (%)	0.619	0.339	0.323	0.359	0.294	0.851	0.744	0.925	0.064		0.027
					RPA						
S1	0.529	0.331	0.177	0.009	0.133	0.462	0.024	0.098	0.047	1.000	0.360
S2	0.166	0.137	1.395	0.033	0.165	0.313	0.074	0.016	0.031	1.000	0.402
S3	0.009	7.382	2.174	0.821	4.936	0.024	0.297	0.150	0.044	1.000	0.240
S4	0.061	8.447	4.401	0.091	4.942	0.214	0.007	0.593	0.054	1.000	0.333
S5	0.084	0.842	7.040	9.732	4.831	0.611	0.500	0.682	0.171	1.000	0.229
S6	0.108	0.405	1.249	3.940	4.085	0.003	0.013	0.400	0.059	1.000	0.530
S7	0.131	2.043	8.178	3.990	6.372	0.180	0.383	0.746	0.060	1.000	0.702
S8	0.232	2.255	10.585	5.236	42.223	13.023	2.589	0.458	0.327	1.000	0.273
S9	1.355	0.946	0.741	0.017	1.776	0.077	0.032	0.567	0.396	1.000	0.460
S10	0.270	0.748	0.202	0.144	1.196	1.565	0.568	0.249	0.057	1.000	0.249

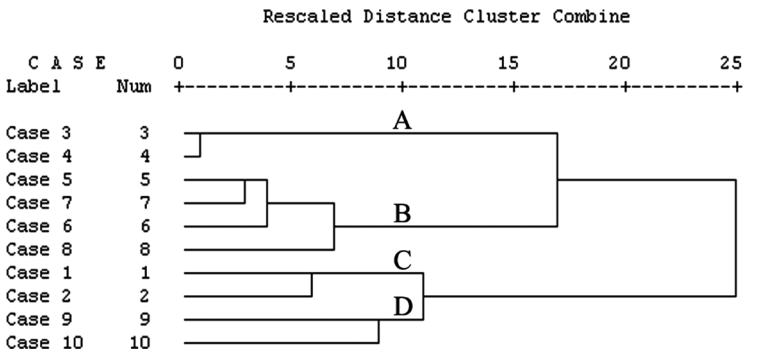

FIGURE 2 Results of hierarchical cluster analysis of ten different animal horn samples rescaled distance cluster combine.

could be classified into four groups (A, B, C, and D) if the Euclidean distance was equal to 10. TAH and AH were in group A; YH-1, YH-2, YH-3, and GH were in group B; WBH and WBHC were in group C; and RH-1 and RH-2 were in group D. The HCA results were consistent with the species taxonomy. However, WBH and RH could be classified into one group if the Euclidean distance was equal to 15, which suggested that WBH and RH were similar and it might be feasible to use WBH as the substitution of RH.

CONCLUSION

In this paper, a simple and reliable LC fingerprint method was developed for classification of different animal horns, which possessed high precision, repeatability and stability. The results of HCA suggested that ten horn samples could be classified into four major groups, which mainly consisted with species taxonomy. The results of HCA also suggested that WBH and RH were close and indicated that WBH was the most similar to RH in comparison with other horns. Quantification of marker constituent HP suggested that the different horn samples shared a similar LC fingerprint and variations in the amounts of marker constituents. In addition, the HP content of RH was almost ten times higher than WBH, which implied that the main chemical constituents' content of RH might be higher than WBH. Thus, it suggested that the dose of WBH used as substitution of RH would be increased by ten-fold, which was consistent with the clinical dose of WBH when used as the substitution of RH in clinical applications. Therefore, it illustrated that the LC fingerprint method and HCA could be used to classify horn-derived TCM, and provide a utility mrthod for the substitution search of precious horn-derived TCM, such as RH and AH.

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