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Identification and Quality Evaluation of Velvet Antler by DNA Barcoding and Stable Isotope Techniques Combined with Chemometrics

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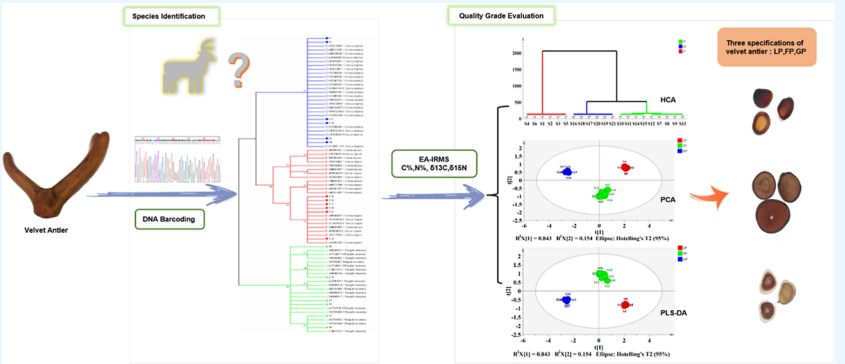

ABSTRACT: This study aimed to identify Velvet antler and its counterfeits and to further evaluate their quality. Mitochondrial cytochrome *b* (Cytb) was used as a target gene to identify Velvet antler samples, and a DNA barcoding method was established for species origin identification in Velvet antlers. After identification, the stable isotope contents and ratios were adopted to evaluate the quality of different specifications of authentic Velvet antler in combination with chemometrics. Two stable isotope contents (C % and N %) and ratios (δ^{13}C and δ^{15}N) in three kinds of Velvet antler slices of different specifications, namely, wax slices, powder slices, and bone slices, were determined. Nine Velvet antler samples sold in the market were identified for label conformity. Only two samples were consistent with the labeled species, and the others were counterfeits. The three slices of Velvet antler of different specifications were clearly distinguished by principal component analysis and hierarchical cluster analysis. Then, the discriminant model of partial least squares discriminant analysis was established, and 100% discrimination accuracy was observed in this model. All the Velvet antler slice samples of different specification samples were grouped clearly according to their sources. In summary, it is feasible for the identification and quality grade evaluation of Velvet antler by DNA barcoding based on mitochondrial Cytb and stable isotope techniques combined with chemometric analysis. The establishment of this method also provided a reference for the evaluation of other animal-derived medicinal materials.

1. INTRODUCTION

As a valuable animal-derived traditional medicinal material (TMM), Velvet antler is officially documented in the Chinese Pharmacopoeia and derived from the nonossified and densely hairy young horns of male sika deer or red deer.[1] It has been widely used for over 2000 years with extensive pharmacological activities and functions for medical care, including the regulation of the immune system, blood system, bone metabolism, and glucose metabolism, as well as anticancer, anti-inflammatory, antioxidant, antifatigue, antibacterial, antivirus, antistress, and analgesic properties.[2,3] To date, Velvet antlers derived from sika deer and red deer have been designated as the authentic medicinal varieties in the world, especially in China, Japan, and South Korea.[3,4]

Nevertheless, due to the diversity of deer species, high medicinal value, and remarkable price differences among different varieties, there is an endless stream of fake Velvet antlers in the market. To pursue a pecuniary advantage, some unscrupulous businessmen have seriously disrupted the market of TMMs by passing shoddy goods off as goods and passing the adulterates for the true commodities in the sale of Velvet antler.

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Because of the special trait of drugs, these problems will lead to unpredictable serious consequences, such as public health risks, not just simple commercial fraud. Therefore, it is urgent to develop reliable ways to identify the authenticity of Velvet antler for ensuring its safety and efficacy of medication.

The identification of Velvet antler depends mainly on morphological identification, microscopic examination, and physical and chemical analyses. However, these traditional methods exhibit some disadvantages, which are particularly vulnerable to the influence of identification experience, medicinal material properties, processing methods, and other factors.[5] Recently, with the rapid development of molecular biological technology, DNA barcoding has been widely used for the identification of animal species due to its outstanding specificity and sensitivity, as well as the advantage that it is not affected by the course of working.[6,7]

As is known, the source and grade of Velvet antler slices affect their price and efficacy.[8] From the tip down, Velvet antler is divided into quality grades of wax, powder, and bone slices. Thus, the quality of Velvet antler slices with different specifications cannot be accurately evaluated by the DNA barcoding technique. At present, spectroscopy and liquid/gas chromatography coupled to mass spectrometry[9−16] are commonly utilized for the quality evaluation of Velvet antlers. Although liquid chromatography is simple, fast, sensitive, and accurate, it is still time-consuming, and many organic reagents are required. Spectral analysis is faster, simpler, and more eco-friendly than liquid chromatography, whereas its sensitivity and specificity still need to be improved. As an ideal candidate, stable isotope technique is widely used in the species identification and origin tracing of agricultural products. It has been gradually adopted to identify the species and geographical origin of TMMs with high sensitivity and low detection limit through evaluating stable isotope contents and ratios in samples,[17−19] whereas this method has not been reported for the quality evaluation of animal-derived TMMs.

Thus, in this study, DNA barcoding and stable isotope techniques are combined for the first time to identify the authenticity of Velvet antlers based on mitochondrial cytochrome *b* (Cytb) gene barcoding and then to evaluate their quality by using stable isotope techniques, as well as their grade with the help of chemometrics. This study not only provides a robust basis for the clinical application of Velvet antler but also provides a reliable method and model reference for the identification and quality evaluation of more animal-derived medicinal materials.

2. EXPERIMENTAL SECTION

2.1. Materials. The QIAamp DNA Investigator Kit (56504) was purchased from Kaijie Enterprise Management (Shanghai) Co., Ltd. DL2000 DNA Maker (0GD20901), agarose (1GA215401), and Ts-GelRed (0GM31401) were obtained from Tsingke Biological Co., Ltd. (Chengdu, China). 2 × Taq PCR MasterMix II (W9525) was bought from Tiangen Biotech (Beijing) Co., Ltd. L14724/H15149 PCR primers were synthesized by Tsingke Biological Technology Co., Ltd. (Chengdu, China). All experiments were approved by the Experimental Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine on July 22, 2020, under the protocol number 2020-25.

2.2. Identification Based on DNA Barcoding. *2.2.1. Sample Collection.* A total of 18 batches of Velvet antler samples (Y1-Y18) were collected, including 6 sika deer antlers, 6 red deer antlers, and 6 reindeer antlers. Among them, 4 sika deer antlers, 3 red deer antlers, and all reindeer antlers were provided by Chengdu Jingbo Biotechnology Co., Ltd. (Chengdu, China), and the rest samples were provided by the Institute of Special Animal and Plant Sciences of CAAS (Changchun, China). In addition, 9 samples labeled authentic Velvet antler were purchased from different medicinal markets and pharmacies in Chengdu and Mianyang (China). These samples were dry slices, and some of them did not have complete morphological characteristics, which were difficult to identify by traditional microscopic identification and thin layer chromatography.

2.2.2. DNA Extraction, Amplification, and Sequencing. All Velvet antler samples were smashed for DNA extraction with a QIAamp DNA Investigator Kit. DNA purity was confirmed using a NanoPhotometer N60 spectrophotometer (IMPLEN Tech., GER). Primers L14724 and H15149[20] were used to amplify the gene sequence in the Cytb region from the extracted DNA. PCR amplification was performed in a 20 μL volume containing 10 μL of 2 × Taq PCR MasterMix, 1 μL of each primer (10 μM), 1 μL of DNA template, and 7 μL of ultrapure water. The PCR conditions were listed as initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR was conducted by using an Eppendorf gene amplification instrument (AG 22331 Hamburg, Eppendorf Co., Ltd, GER). The PCR-amplified products were separated in a 2% (w/v) agarose gel containing 5 μL of GelRed. Then, they were sequenced.

2.2.3. Sequence Analysis. The bidirectional sequencing results were manually corrected and spliced using Chromas and DNAMAN programs (Table S1), and the low-quality regions at both ends of the spliced sequences are removed. The spliced sequences were submitted to the National Center for Biotechnology Information of U.S. for BLAST analysis to determine their accuracy. The Cytb gene sequences of Velvet antler were downloaded from the GeneBank database as references and then compared and aligned by MEGA 7.0 software. A neighbor-joining (NJ) tree was constructed by the K2P model, and the reliability of phylogenetic analysis was evaluated by MEGA 7.0.

2.3. Stable Isotope Analysis. *2.3.1. Sample Collection and Pretreatment.* Twenty-one sika Velvet antlers were provided by Chengdu Jingbo Biotechnology Co., Ltd. (Chengdu, China). All samples were dried slices. The dry samples were crushed into powder with a pulverizer, and the powder was sieved through a 125 μm sieve and then stored in a desiccator.

2.3.2. Stable Isotope Ratio Analysis. All samples for stable isotope ratio analysis were weighed on an M5X analytical balance (METTLER TOLEDO International Co., Ltd, Switzerland). A total of 1.00 mg of each sample was transferred into a small tin cap for carbon and nitrogen isotope analysis. The C %, N %, δ^{13}C ‰, and δ^{15}N ‰ values of all samples were analyzed by a Vario EL III-Isoprime EA-IRMS (Elementar, Germany). Helium (He) was used as a carrier and reference gas for elemental analysis. Carbon was oxidized to CO_2 by combustion at 1150 °C. In this process, the nitrogen in the samples was converted into nitrogen oxide and then reduced to N_2 at 810 °C. Subsequently, the products were detected by isotope ratio mass spectrometry. CO_2 and N_2 were used as reference gases for mass analysis. USGS40 ($\delta^{13}C_{V\text{-}PDB}$ = −26.39‰, $\delta^{15}N_{AIR}$ = −4.52‰) and casein ($\delta^{13}C_{V\text{-}PDB}$ = −26.98‰, $\delta^{15}N_{AIR}$ = 5.94‰) were chosen as the carbon and nitrogen isotope standard materials.

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The stable isotope ratio was calculated according to the following equation[21,22]

$$\delta\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000 \quad (1)$$

where δ (‰) represents the difference between the sample and international standard. R is the ratio of the heavier isotope to the lighter isotope, namely, $^{13}C/^{12}C$ and $^{15}N/^{14}N$.

2.4. Statistical Analysis. The data were analyzed by using SPSS 24.0 statistical software (SPSS Inc., USA) and SIMCA-P 14.1 software (Umetrics, Sweden). Hierarchical clustering analysis (HCA), principal component analysis (PCA), and partial least squares discriminant analysis (PLS-DA) were implemented by using the SIMCA-P 14.1 software (Umetrics, Sweden), which were used for data visualization, sample classification, and prediction model establishment.

3. RESULTS AND DISCUSSION

3.1. Identification of Velvet Antler Using a Cytb Sequence-Based PCR Assay. A Cytb sequence-based PCR assay was introduced for the identification of Velvet antlers. The DNA of all samples was extracted successfully. The amplification length of the Cytb target fragment in these Velvet antler samples was approximately 460 bp by PCR, which is shown in Figure 1.

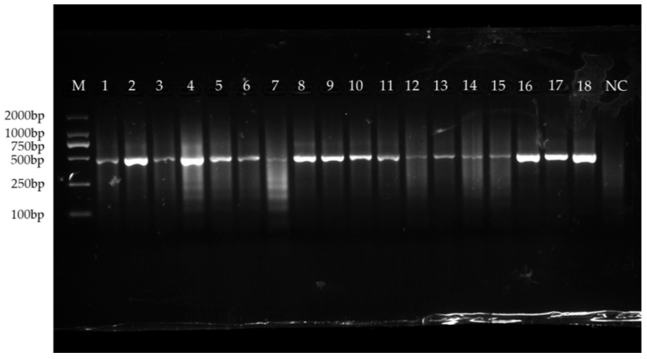

Figure 1. Electrophoresis graph of PCR products of 18 Velvet antler samples.

High-quality gene sequences were obtained after sequencing all PCR products. By comparison with the GenBank database, the sequence similarity of the 18 Velvet antler samples was above 97.5%. Studies have shown that the genetic distance of Cytb segregation in the same species is less than 2.5%.[23] Therefore, when comparing the sequencing results of Velvet antler samples, if the sequence similarity of Cytb was greater than 97.5%, it could be determined to be the same species. The BLAST results confirmed that the identification results of 18 Velvet antler samples were consistent with the species information obtained during sample collection. The sample information and their DNA identification results (GenBank Accession) are listed in Table 1.

3.2. NJ Tree Analysis. The Cytb sequences of sika deer, red deer, and reindeer were downloaded from the GenBank database and compared with the 18 Cytb sequences amplified in this study. According to the comparison results, the molecular phylogenetic tree was constructed by using the (NJ) method with 1000 bootstrap repetitions to evaluate the identification ability of DNA barcoding for antler deer species. Bootstrap values less than 50% were hidden. The NJ tree in Figure 2 showed that the collected sika deer antler samples and sika deer species were clustered into one branch, red deer antler samples

Table 1. Species Identification of Velvet Antler Samples Based on DNA Barcoding (GenBank Accession)

no.	species name	percent identity (%)	GenBank accession
Y1	*Cervus elaphus macneilli*	100	KX449334.1
Y2	*Cervus elaphus macneilli*	98.48	KX449334.1
Y3	*Rangifer tarandus*	100	MG765424.1
Y4	*Cervus elaphus*	99.57	AB245427.2
Y5	*Cervus elaphus macneilli*	99.56	KX449334.1
Y6	*Rangifer tarandus granti*	98.06	AY726728.1
Y7	*Rangifer tarandus granti*	100	AY726728.1
Y8	*Rangifer tarandus granti*	99.27	AY726728.1
Y9	*Rangifer tarandus granti*	100	AY726728.1
Y10	*Cervus elaphus macneilli*	100	KX449334.1
Y11	*Cervus elaphus macneilli*	100	KX449334.1
Y12	*Cervus nippon hortulorum*	99.35	KR868807.1
Y13	*Rangifer tarandus*	100	DQ673133.1
Y14	*Cervus nippon hortulorum*	99.78	KR868807.1
Y15	*Cervus nippon hortulorum*	99.78	KR868807.1
Y16	*Cervus nippon hortulorum*	100	KR868807.1
Y17	*Cervus nippon hortulorum*	100	KR868807.1
Y18	*Cervus nippon hortulorum*	100	KR868807.1

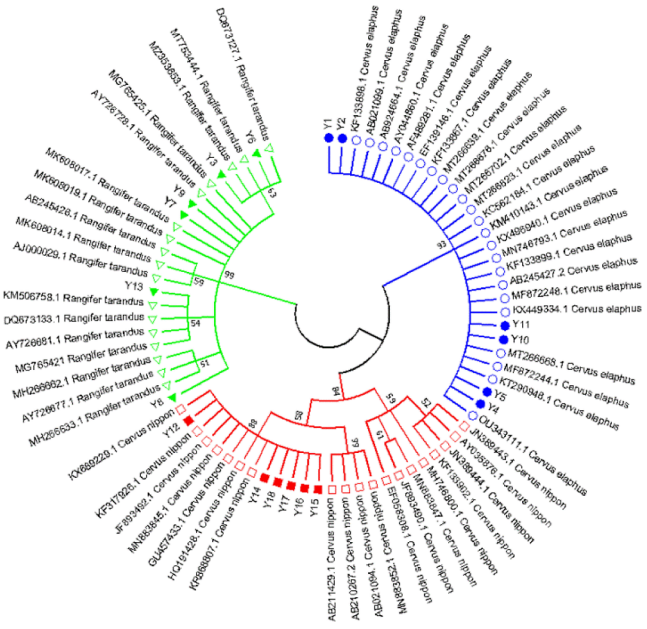

Figure 2. NJ phylogenetic tree of deer species based on the sequence of Cytb. Note: the bootstrap value (>50%) is displayed above the relevant branch. The sample number is consistent with that in Table 1.

and red deer species were grouped into one branch, as well as reindeer antler samples and reindeer species were classed into another branch. The results indicated that the species clustering results of the collected Velvet antler samples were consistent with the collected deer species information. It further confirmed that DNA barcoding based on the Cytb sequence could be used for the identification of Velvet antlers.

3.3. Identification of Commercial Velvet Antler Samples. The established DNA barcoding technology was used to identify the species of commercial Velvet antler samples, and the label compliance of Velvet antler commodities on the market was analyzed. The identification results showed that the 9 commercial Velvet antler products could be identified as clear species. Comparing the species identified by DNA barcoding

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with the tagged species, it was found in Table 2 that 7 samples were inconsistent with the DNA barcoding identification results among the 9 commodities labeled as authentic Velvet antlers, and only two red deer antler samples were consistent with the tagged species. It indicated that the proportion of nonauthentic Velvet antler was very high, and these varieties mainly originated from reindeer, which had seriously disturbed the Velvet antler market.

Table 2. Authenticity Identification of Commercial Velvet Antlers Based on the DNA Barcoding Technique

no.	label name	GenBank	percent identity (%)	label match or not
1	red deer antler slice	*Cervus elaphus macneilli*	99.78	
		Cervus elaphus hippelaphus	99.76	yes
		Cervus elaphus hispanicus	99.76	
		Cervus elaphus scoticus	99.76	
2	sika deer antler slice	*Elaphurus davidianus*	100	no
3	sika deer antler slice	*Capreolus capreolus*	99.78	no
		Capreolus pygargus tianschanicus	99.56	
4	sika deer antler slice	*Rangifer tarandus terraenovae*	99.53	
		Rangifer tarandus caribou	99.75	no
		Rangifer tarandus granti	100	
		Rangifer tarandus groenlandicus	99.75	
5	sika deer antler slice	*Rangifer tarandus terraenovae*	99.29	
		Rangifer tarandus caribou	99.06	
		Rangifer tarandus granti	99.76	no
		Rangifer tarandus groenlandicus	99.51	
6	sika deer antler slice	*Rangifer tarandus terraenovae*	99.29	
		Rangifer tarandus caribou	99.52	no
		Rangifer tarandus granti	99.75	
		Rangifer tarandus groenlandicus	99.75	
7	red deer antler slice	*Cervus elaphus sibericus*	100	
		Cervus elaphus songaricus	100	
		Cervus elaphus xanthopygus	99.13	yes
		Cervus elaphus kansuensis	98.91	
		Cervus elaphus alxaicus	98.70	
8	sika deer antler slice	*Rangifer tarandus terraenovae*	99.06	
		Rangifer tarandus caribou	99.28	no
		Rangifer tarandus granti	99.51	
		Rangifer tarandus groenlandicus	99.51	
9	sika deer antler slice	*Rangifer tarandus terraenovae*	99.06	
		Rangifer tarandus caribou	100	no
		Rangifer tarandus granti	99.52	
		Rangifer tarandus groenlandicus	100	

3.4. C, N Isotope Analysis. Here, the contents of two stable isotope (C % and N %) and ratios (δ^{13}C and δ^{15}N) from three Velvet antler slices of different specifications were determined, which have been presented in Figure 3 and Table 3. Significant differences in the contents and ratios of stable isotopes among the three Velvet antler slices of different specifications could be clearly observed. The isotope contents of LP (wax slices) samples were the highest, followed by FP (power slices) and GP (bone slices) samples. The same trends were observed regarding the isotope ratios. These results indicated that the three Velvet antler slices of different specifications presented the characteristic distribution of stable isotope contents and ratios, which was in line with the growth regularity of Velvet antler. From the tip to the bottom, the content of inorganic elements gradually increased, and the content of organic elements gradually decreased with increasing ossification.[24,25]

3.5. Chemometric Analysis. *3.5.1. Hierarchical Cluster Analysis.* Hierarchical cluster analysis (HCA) of the Velvet antler samples was performed to distinguish the three Velvet antler slices of different specifications based on differences in two stable isotope contents and ratios. The data sets were treated by using the squared Euclidean distance as a similarity measurement. Figure 4 shows that all the LP, GP, and FP samples were clustered exactly. All samples were clustered into two clusters: the first cluster consisted of LP samples only, and the second cluster consisted of FP and GP samples. Then, the second cluster was then divided into two groups: FP samples in one group and GP samples in the other group. All the samples were clustered correctly with a cutoff distance of 500. This result indicated that stable isotope contents and ratios combined with HCA could be used to well distinguish the three kinds of Velvet antler slices of different specifications.

3.5.2. Principal Component Analysis. PCA is an unsupervised method that does not depend on the labels for classification, which converts multiple variables into a few new comprehensive variables to describe the characteristics of the original data on the basis of retaining the original information.[26] Here, PCA was implemented based on the data of C, N %, δ^{13}C, and δ^{15}N. It could be found that only the first principal component (PC1) could account for 85.6% of the total variance, and the second principal component (PC2) could explain 13.7%. The cumulative contribution rate of these two principal components' variance was 99.3%, which could explain almost all the original information. Figure 5 indicates that the LP, GP, and FP Velvet antler slice samples of different specification samples were grouped clearly, which was consistent with the findings of HCA.

3.5.3. Partial Least Squares Discrimination Analysis. PLS-DA is a commonly used supervised multivariate statistical analysis method. Classifying each group is more conducive to explore the similarity and difference between multi-group samples than PCA.[27] Twenty-one samples were divided into 17 correction sets and 4 verification sets by using the SPSS 24.0 software. A PLS-DA model was established, in which the number of principal components was 3. The parameters of the PLS-DA model were as follows: $R^2X = 0.997$; $R^2Y = 0.983$; $Q^2 = 0.981$. Generally, the model was considered to have excellent predictability when R^2 and Q^2 were close to 1 and the gap was less than 0.3.[27]

The score plot of PLS-DA is shown in Figure 6. It could be clearly found that the samples of wax slices, powder slices, and

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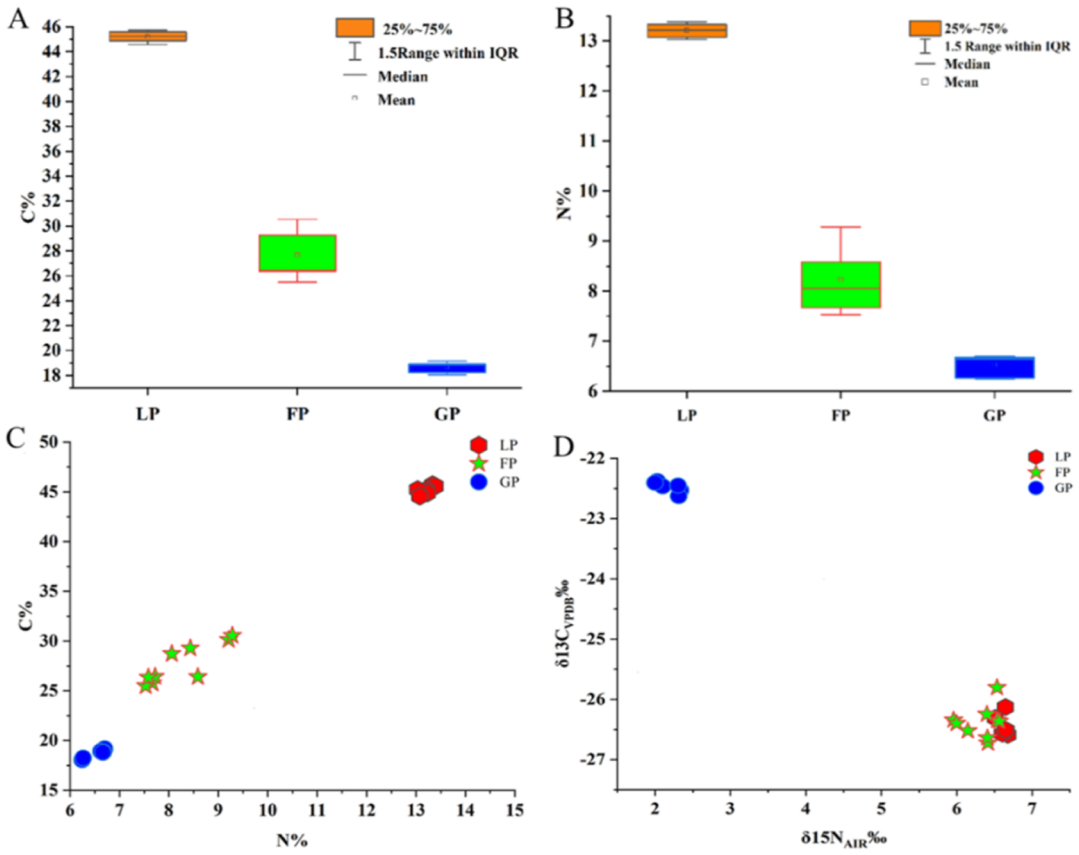

Figure 3. Boxplots of (A) C % and (B) N % of 3 Velvet antler slices of different specifications. Scatter plots of (C) C % and N % and (D) δ^{13}C and δ^{15}N of 3 Velvet antler slices of different specifications. LP, FP, and GP represent wax, powder, and bone slices of Velvet antler of different specifications, respectively.

Table 3. Stable Isotope Contents and Ratios from Three Velvet Antler Slices of Different Specifications[a]

	LP (n = 6)			FP (n = 9)			GP (n = 6)		
element	mean	min	max	mean	min	max	mean	min	max
δ^{13}C	−26.44	−26.59	−26.13	−26.38	−26.73	−23.93	−22.48	−22.63	−22.38
δ^{15}N	6.62	6.51	6.68	6.33	2.7	6.41	2.18	2	2.34
C %	45.2	45.22	45.71	27.68	25.49	29.28	18.67	18.06	19.16
N %	13.21	13.03	13.38	8.23	7.27	8.43	6.528	6.24	6.69

[a]LP, FP, and GP represent wax, powder, and bone slices of Velvet antler of different specifications, respectively.

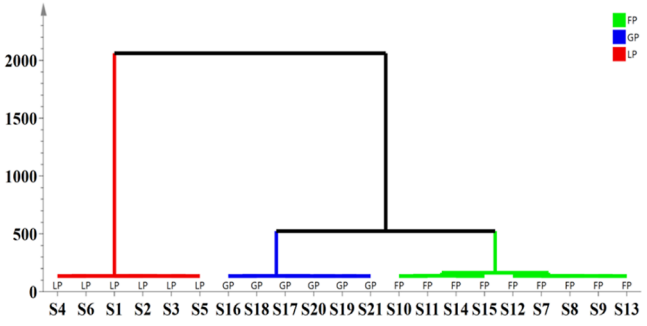

Figure 4. HCA dendrogram of three Velvet antler slices of different specifications derived from their stable isotope contents and ratios.

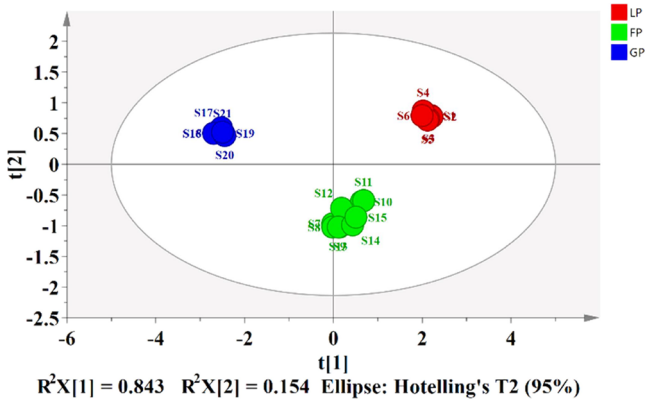

Figure 5. Score plot of PCA based on stable isotope contents and ratios.

bone slices of Velvet antler were distributed in three independent spaces, which was consistent with the PCA results. A permutation test was performed to verify whether the PLS-DA model was overfitting, and the points of the y-axis intercepted by R^2 and Q^2 were less than 0.3 and 0.05, respectively,[28,29] indicating that this model was not overfitting. Moreover, external validation was carried out using four samples outside the model to evaluate the accuracy of the established model. The

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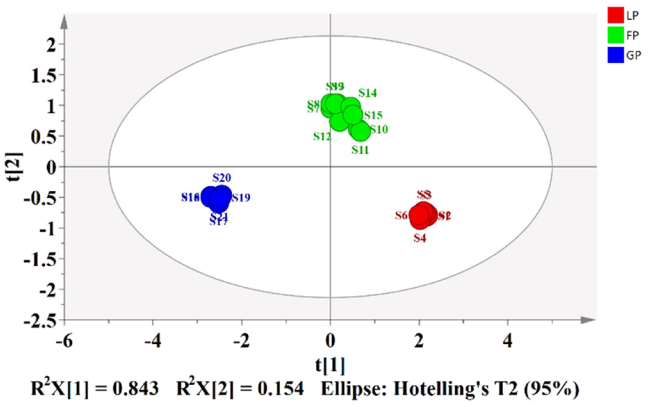

Figure 6. Score plot of PLS-DA based on stable isotope contents and ratios.

results showed that the four samples were correctly classified with an accuracy of 100%. As shown in Figure 7, variable importance projection (VIP) analysis was also performed. The variables with VIP > 1 were N % and δ^{13}C, indicating that they exhibited greater effects on model grouping.

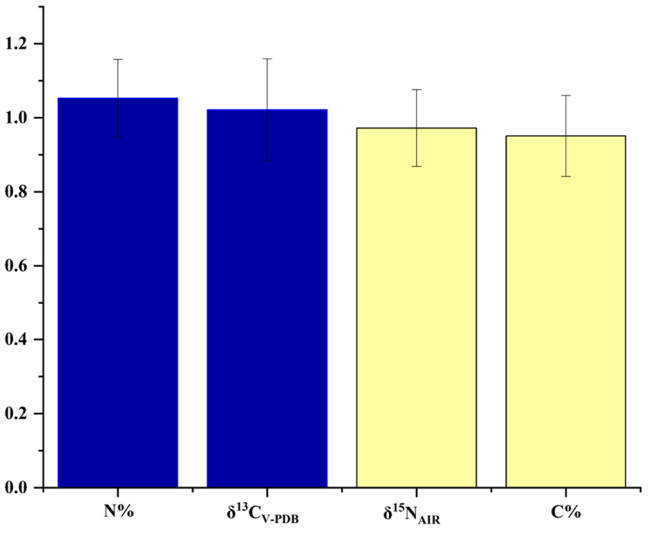

Figure 7. VIP plot of PLS-DA to indicate the importance of the variables.

In summary, it was concluded that the PLS-DA model was stable and reliable. It was feasible to distinguish three kinds of Velvet antler slices of different specifications using stable isotope contents and ratios combined with PLS-DA analysis.

4. CONCLUSIONS

As a precious TMM, Velvet antler has a complex source, high medicinal value, and high price, leading to serious adulteration in the market. The common methods for the authenticity identification of Velvet antler rely either on experience or on the chemical composition of the medicinal material itself, which is easily affected by a series of factors, such as variety, origin, processing, storage environment, and transportation mode.[30] In this study, the DNA barcoding method based on the Cytb sequence was introduced to authenticate the collected Velvet antler samples. The results indicated that (1) the DNA barcoding method could identify Velvet antler samples quickly and effectively and (2) the adulteration of Velvet antler was fairly severe. Due to the chaotic market for Velvet antler medicinal materials, this issue needs to be considered to ensure the safety and effectiveness of drug use; (3) certainly, DNA barcoding based on the Cytb gene sequence was not perfect in every respect. During BLAST comparison, it was found that the Cytb gene fragment amplified by universal primers could accurately identify deer species, yet it was impossible to identify different subspecies of the same deer species. This required designing specific primers and using specific PCR to address it, which was also one of our future research goals.

After identifying the authenticity of Velvet antler, the quality of sika Velvet antler slices of different specifications was evaluated based on stable isotope contents and ratios. As is known, according to different cutting parts and processing methods, Velvet antlers on the market are often divided into wax slices, powder slices, bone slices, and so forth.[31] The quality of Velvet antler slices with different specifications is different, and it is usually considered that the quality of wax slices is the best, followed by powder slices and bone slices. Without a doubt, the resulting price differences are also great. Although Velvet antler is documented in the Chinese Pharmacopoeia, there is no clear division of Velvet antler slice specification. The imperfect quality grade standard of Velvet antler slices makes it difficult to effectively control and evaluate the quality of Velvet antler products. Stable isotope technology was introduced to solve this problem. According to different cutting parts of Velvet antler slices with different specifications, differences in chemical components, and the contents and ratios of C and N stable isotopes in 21 sika Velvet antler slices of different specifications were determined quickly and accurately by EA-IRMS. Then, the grade quality of sika Velvet antler was evaluated by chemometrics. The results showed that stable isotope contents and ratios combined with multivariate statistical analysis could be used as a robust and reliable method for evaluating the grade quality of Velvet antler. The establishment of this method could not only standardize the market for Velvet antler medicinal materials and protect the interest of consumers but also represent a beneficial attempt and exploration of a new model for the quality grade evaluation of TMMs.

In summary, DNA barcoding and stable isotope techniques combined with the chemometrics method were successfully established for the accurate identification and quality evaluation of Velvet antlers for the first time in this study. The strategy could not only accurately and quickly distinguish the quality of Velvet antler but also be used for the quality assessment of other animal-derived medicinal materials, which provided a powerful reference for the overall quality evaluation of TMMs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05173.

Cytb gene fragment sequences spliced by Chromas and DNAMAN programs from 18 Velvet antler samples (PDF)

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