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Establishment and comparative analysis of HPLC fingerprints of deer tissues

Mengjie Yao[A,*], *Haiping Zhao*[A,*], *Xiaoyan Qi*[A], *Yuan Xu*[A], *Wenyuan Liu*[A] *and Chunyi Li*[A,B,C]

[A]State Kay Laboratory for Molecular Biology of Special Economic Animals, 4899 JuYe Street, Nanguan District, Changchun, Jilin130112, China.
[B]Changchun Sci-Tech University, 1699 DongHua Street, Shuangyang District, Changchun, Jilin 130022, China.
[C]Corresponding author. Email: lichunyi1959@163.com

Abstract

Context. With the increasing use of velvet antlers (VA) as functional food or traditional Chinese medicine, the quality control has become more and more important.

Aims. Establish an effective method to provide a way of distinguishing VA from other types of deer tissue.

Methods. In the present study, 18 samples from three types of deer tissue were analysed on the basis of high-performance liquid chromatography, and a chromatogram of each sample was obtained. Then, these chromatograms were processed using the similarity evaluation system for chromatographic fingerprints of traditional Chinese medicine, to give the fingerprints of three deer tissues. The chemometric methods were used to analyse the fingerprint results, so as to identify the three types of deer tissue.

Key results. Shared peaks of VA, venison and deer bone were identified using similarity evaluation system. The results showed that, in total, 19 peaks were identified among these three types of deer tissue. Compared with venison, VA lacked three peaks (Numbers 3, 4 and 17); compared with deer bone, VA had six extra peaks (Numbers 2, 5, 8, 9, 14 and 19). The results of chemometric methods showed that different tissue samples could be classified into three categories by using both cluster analysis and principal component analysis. After principal component analysis and partial least-square discrimination analysis, seven peaks were selected, which had significant influence on the classification of VA, venison and deer bone.

Conclusions. The high-performance liquid-chromatography fingerprints in combination with chemometric methods can be used to effectively distinguish three deer tissue types, namely, VA, venison and deer bone.

Implications. We believe the method offers a useful tool much needed in the current Chinese velvet market.

Additional keywords: deer bone, chemometric methods, velvet antler, venison.

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Introduction

Velvet antler (VA) is a globally renowned valuable traditional Chinese medicine (TCM) and is used to nourish the human body (Liu *et al*. 2015; Zhang *et al*. 2016, 2018). Originated from un-ossified young antler of a male deer (sika deer or wapiti; Commission 2015), VA in China are mainly used to improve kidney function, enrich essence and blood, strengthen muscle and bone, regulate blood vitality and stimulate toxic discharge (Commission 2015; Wang *et al*. 2017). With the increase in health awareness, demand for deep-processed VA products is rising sharply. However, due to quality control for VA products currently lacking, development of the VA industry is seriously hindered. In recent years, a method of DNA molecular biometric identification (Zhang *et al*. 2011; Gao *et al*. 2018) has been considered to be one of the effective ways to identify the authenticity of VA due to high sensitivity of the method. However, DNA method is incapable of discriminating VA from the other types of deer tissue in a product because it is not tissue specific, but rather a species-specific. Hence, DNA detection method cannot be used to accurately identify whether a VA prescription contains a VA component or other types of deer tissue. It is of great importance to establish a reliable way of identifying VA tissue in a product if VA products are to be further developed and deep-processed. It has been reported that HPLC fingerprints can be used to comprehensively reflect chemical composition of complex prescriptions in the TCM, so are often used to evaluate the quality of a type of TCM

[*]These authors contributed equally to this work.

prescription (Chen *et al*. 2015; He *et al*. 2015). Both cluster analysis and principal component analysis (PCA) are being used to analyse HPLC fingerprints (Feng *et al*. 2014; Huang *et al*. 2019). In the present study, VA, venison and deer bone were used to establish HPLC fingerprints, and these were then used to distinguish VA from the two other types of deer tissue. We believe that in combination with DNA detection method, our approach could effectively determine whether the so-claimed VA compounds contain VA components and which deer species they come from. A reliable method is much needed in the current Chinese velvet market for clearance of fake VA products.

Materials and methods

Collection of VA, venison and deer bone

Velvet antler, venison and deer bone from six deer were collected immediately after slaughtering in a deer slaughter plant in Changchun, China, and cleaned, frozen, sliced, dried at 65°C and, finally, ground to powder.

Preparation of tissue-sample solution

A subsample of powdered VA, venison or deer bone from each deer was weighed (0.5 g/tissue), diluted in 10 mL of water in a 50-mL centrifuge tube, which was placed in an ultrasonic bath, ultrasonificated for 30 min (at 50 kHz), and centrifuged (at room temperature, 10 000*g*) for 15 min, the supernatant was collected. Then the residue was extracted once again in the same way as above, and all supernatants were pooled together. Distilled water was added to the tube containing the supernatant to make the final volume of 25 mL. The resultant solution was filtered through a 0.45-μm pore-size membrane, and the filtrate was taken as the sample solution.

Preparation of standard solution

Uracil (Merck Life Science Co., Ltd. Shanghai, China) and hypoxanthine (Merck Life Science Co., Ltd.) were weighed to four digits after decimal point, and distilled water was added to obtain a concentration of 10 μg/mL solution.

Chromatographic conditions and system suitability

Chromatographic analysis was conducted using Waters e2695 system (Waters Corporation, Milford, MA, USA), which was equipped with a 2489UV detector and connected to the Waters Empower 3 data station. Pursuit XRs C18 column (250 × 4.6 mm 5μm) (Agilent Technologies, Santa Clara, CA, USA) was used for chromatographic separation at 30°C. Detection wavelength was set at 260 nm, the flow rate at 0.5 mL/min, and 10-μL sample solution was injected each time. The mobile phase was composed of methanol (solvent A) and water (solvent B), and the gradient elution for the mobile phase was set as follows: 0–42 min, 1 –15% A. Under these chromatographic conditions, the theoretical number of plates is not fewer than 10 000 for uracil, not fewer than 15 000 for hypoxanthine, the resolution is greater than 1.5, and the USP trailing factor ranges from 0.95 to 1.13. The standard solution was measured five times continuously, the relative standard deviation (RSD) of the uracil peak area was 0.168%, and the RSD of the hypoxanthine peak area was 0.220%.

Validation of HPLC method

Precision of our HPLC method was verified through running the same sample consecutively for five times. Reproducibility of the method was evaluated by comparing the results of running the same samples for five times (independently prepared.) Sample stability was monitored through analysing the same sample solutions for 1 day at an interval of 6 h. Using hypoxanthine as a reference peak (hypoxanthine was found to have a greater resolution and a better peak shape than was uracil in the present study), the RSD of the relative retention time and relative peak area of the common peaks of the chromatogram obtained in each experiment were calculated. The value of RSD was taken as the appraisal index.

HPLC fingerprints

According to the above-described HPLC method, the sample solutions of VA, venison and deer bone were sequentially analysed, and the chromatogram of each sample was obtained. These chromatograms were processed using similarity evaluation system (SES, 2004A, China Pharmacopoeia Committee, China).

Cluster analysis

The cluster-analysis method was used to analyse the characteristic data of each sample and to group the samples according to the degree of closeness in the nature of the samples. This method can provide an objective basis for the classification of TCM (Pang *et al*. 2016; Shan *et al*. 2018; Sun *et al*. 2017). The peak area of each sample was processed by SPSS 23.0 software (SPSS, Armonk, NY, USA) for cluster analysis in the present study.

PCA and partial least-square discrimination analysis (PLS-DA)

Currently, PCA is one of the most commonly used unsupervised analysis methods. This technique can reduce the dimensionality of the original data set by explaining the correlation among a large number of variables in terms of a smaller number of underlying factors (Sun *et al*. 2014; Guo *et al*. 2019). PLS-DA is a supervised statistical method for discriminant analysis. The method uses a partial least-square regression to establish a relationship model between the peaks and the sample class, so as to achieve prediction of the sample class. At the same time, the influence of each peak on the classification of each group of samples is measured by calculating variable importance for the projection (VIP). SIMCA-14.1 software (Umetrics, Umea, Sweden) was adopted for analysis in both PCA and PLS-DA.

Results

HPLC method validation

The validation results of HPLC method are shown in Tables 1–3; the method had good precision and repeatability, and the sample solution was found to be stable at least within 24 h.

Table 1. Results of precision test

Number	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
			Relative retention time				
1	0.2965	0.6633	0.9088	1	1.2164	1.8641	1.9799
2	0.2976	0.6659	0.9095	1	1.2188	1.8602	1.9885
3	0.2998	0.6699	0.9154	1	1.2262	1.866	1.982
4	0.2977	0.6683	0.9054	1	1.2218	1.8629	1.9806
5	0.2965	0.6653	0.9056	1	1.218	1.8607	1.9691
RSD (%)	0.453	0.389	0.446	0	0.317	0.129	0.353
			Relative peak area				
1	0.1708	0.5217	0.8999	1	1.3836	1.8675	0.9971
2	0.164	0.5123	0.8939	1	1.3655	1.8504	0.9977
3	0.164	0.5059	0.9034	1	1.3734	1.797	0.9933
4	0.1643	0.504	0.9291	1	1.3971	1.7877	1.0075
5	0.1618	0.4946	0.8993	1	1.3747	1.7462	1.0069
RSD (%)	2.064	1.984	1.528	0	0.874	2.717	0.634

Table 2. Results of reproducibility test

Number	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
			Relative retention time				
1	0.2963	0.6669	0.9099	1	1.2142	1.84	1.9541
2	0.2962	0.6683	0.9104	1	1.2084	1.8438	1.9579
3	0.297	0.6684	0.9069	1	1.2116	1.8442	1.9636
4	0.298	0.6679	0.9089	1	1.2105	1.8649	1.9713
5	0.2982	0.6701	0.9064	1	1.2082	1.856	1.9589
RSD (%)	0.314	0.173	0.196	0	0.205	0.56	0.337
			Relative peak area				
1	0.1422	0.4463	0.8522	1	1.3092	1.5773	0.9434
2	0.1402	0.4471	0.8683	1	1.3345	1.5743	0.9658
3	0.1358	0.4268	0.8454	1	1.2922	1.5056	0.9375
4	0.1349	0.4347	0.8572	1	1.3126	1.5217	0.951
5	0.1324	0.4291	0.8048	1	1.3027	1.5062	0.9427
RSD (%)	2.923	2.172	2.871	0	1.193	2.342	1.162

Table 3. Results of stability test

Number	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
			Relative retention time				
1	0.2965	0.6633	0.9088	1	1.2164	1.8641	1.9799
2	0.2965	0.6653	0.9056	1	1.218	1.8607	1.9691
3	0.3002	0.6702	0.9164	1	1.2314	1.8735	1.9911
4	0.2973	0.6645	0.9083	1	1.2211	1.8577	1.9845
5	0.2982	0.6677	0.9077	1	1.223	1.8649	1.9841
RSD (%)	0.518	0.414	0.453	0	0.48	0.319	0.41
			Relative peak area				
1	0.1708	0.5217	0.8999	1	1.3836	1.8675	0.9971
2	0.1618	0.4946	0.8993	1	1.3747	1.7462	1.0069
3	0.165	0.5054	0.9198	1	1.3718	1.7958	0.9935
4	0.1668	0.5119	0.8933	1	1.3694	1.8484	0.9973
5	0.1639	0.5022	0.9272	1	1.3915	1.7821	1.004
RSD (%)	2.05	2.019	1.62	0	0.666	2.739	0.551

HPLC fingerprints

The HPLC fingerprints of three types of deer tissue are shown in Fig. 1. The reference fingerprints of VA, venison and deer bone are shown in Fig. 2, and retention time and area of matched peaks of 18 samples are shown in Table 4. Overall, our results showed that VA lacked three peaks (Numbers 3, 4 and 17) when compared with venison; but had six extra peaks (No. 2, 5, 8, 9, 14 and 19) when compared with deer bone. The similarity analysis between samples was performed using similarity evaluation-system software and HPLC chromatogram of VA samples was used as the reference. The results (Table 5) show that the similarity range between VA samples was 0.891–0.995 (intra-group), whereas the range between venison and VA samples was 0.469–0.796 (inter-group); the range between deer bone and VA samples was 0.574–0.82 (inter-group). Out results suggest that similarity of the intra-group is much higher than that of the inter-group; that is, the three deer tissues could be clearly separated by the similarity analysis.

Cluster analysis

The results of cluster analysis clearly divided the samples into three categories. Samples S1–S6 were from VA, being grouped together, Samples S7–S12 were from venison, being grouped together, and Samples S13–S18 from deer bone, and they were grouped together (Fig. 3). Our results, convincingly, demonstrated that samples from the same tissue had a greater similarity, whereas those from different types of deer tissue had fewer similarities.

PCA and PLS-DA analysis

Principal component analysis was performed using the peak area data of HPLC fingerprints to generate an array (18 × 19 data matrix), in which each row represents one sample and each column represents the area of each chromatographic peak. The eigenvalues of the first and second principal components (PC1 and PC2) were all >1, and 78.9% of the

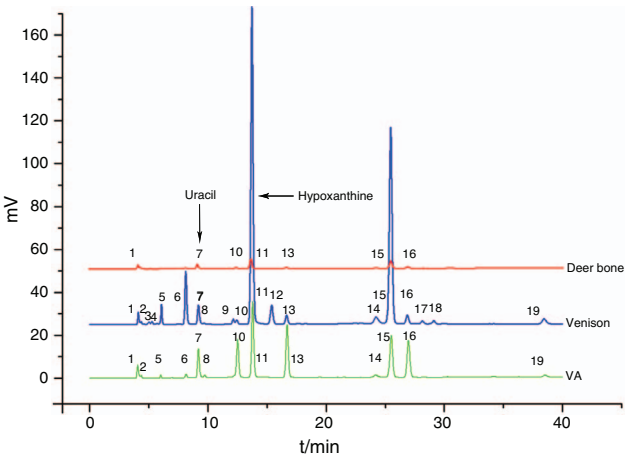

Fig. 2. Reference fingerprints of velvet antler (VA), venison and deer bone. The fingerprints were generated on the basis of the high-performance liquid-chromatography (HPLC) fingerprints. Note that VA lacked three peaks (Numbers 3, 4 and 17) compared with venison, but had six extra peaks (Numbers 2, 5, 8, 9, 14 and 19) compared with deer bone.

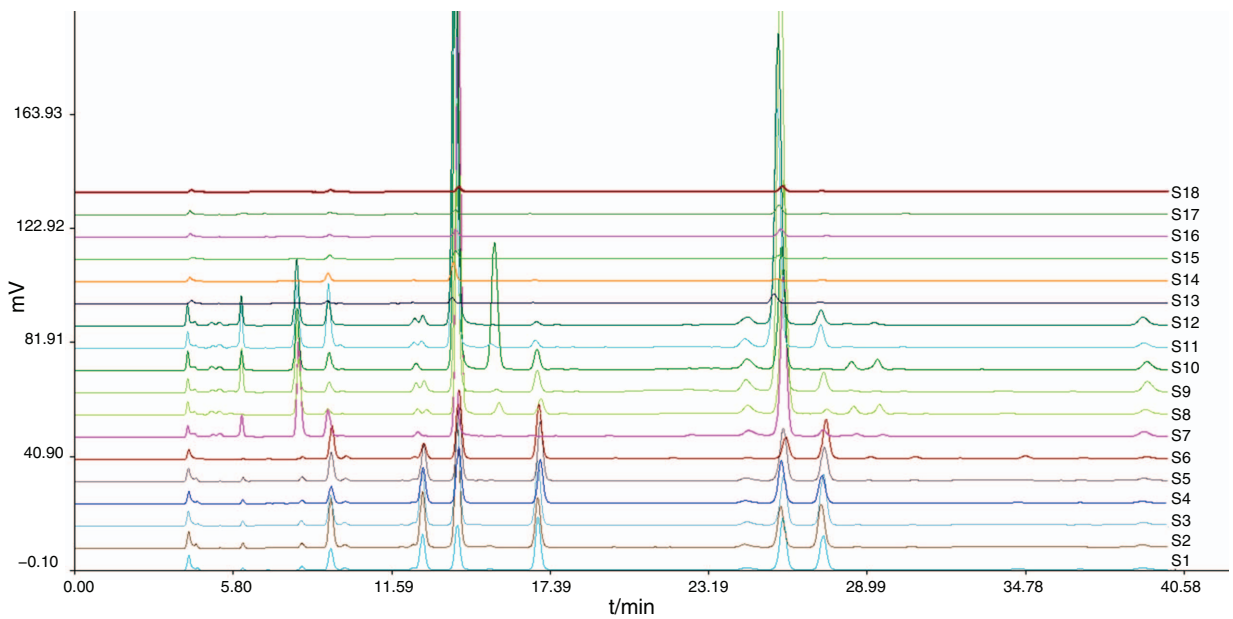

Fig. 1. High-performance liquid-chromatography (HPLC) fingerprints of the 18 samples from three types of deer tissue. The fingerprints were generated through processing the chromatograms of the three types of deer tissue using similarity evaluation-system (SES) software. Samples S1–S6 are from velvet antler (VA), Samples S7–S12 from venison and Samples S13–S18 from deer bone. Note that the fingerprints from each tissue type had characteristic profiles, but were highly different from the other tissue types.

Table 4. Retention times and peak areas of the 18 samples

Peak number	Time	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18
Peak 1	4.149	50.35	53.543	50.412	41.08	45.549	30.925	32.334	35.051	37.614	49.554	45.683	55.849	11.812	15.474	6.797	12.237	11.161	9.479
Peak 2	4.393	6.823	9.699	6.52	6.213	8.84	0	7.48	7.531	9.127	8.007	6.097	10.125	0	0	0	0	0	0
Peak 3	5.028	0	0	0	0	0	0	8.974	14.519	10.215	14.907	7.903	12.82	0	0	0	0	0	0
Peak 4	5.292	0	0	0	0	0	0	13.003	10.922	13.903	12.77	14.728	12.278	0	0	0	0	0	0
Peak 5	6.122	5.554	13.343	11.96	10.378	10.017	1.632	52.276	0	109.608	45.455	110.288	76.055	0	0	0	0	0	0
Peak 6	8.221	13.797	17.111	18.2	11.275	16.23	7.546	376.148	212.965	260.301	236.339	243.618	250.118	0	0	0	2.25	2.192	1.435
Peak 7	9.27	88.031	201.906	125.782	69.314	119.769	139.05	111.269	21.841	41.76	69.389	254.015	96.14	9.233	32.211	15.871	8.987	12.971	10.355
Peak 8	9.823	8.261	14.079	13.958	7.542	11.318	12.519	5.989	3.349	5.777	0	12.132	0	0	0	0	0	0	0
Peak 9	12.447	6.444	9.931	8.723	4.412	7.663	7.466	17.84	23.84	40.231	29.979	23.474	30.367	0	0	0	0	0	0
Peak 10	12.733	161.909	255.852	253.365	164.663	164.838	70.145	0	22.565	54.939	0	31.601	44.684	4.03	4.393	0	2.131	4.198	2.755
Peak 11	13.817	203.5	549.791	474.389	257.927	345.546	320.997	2188.132	1535.731	971.439	2438.305	1701.729	2644.821	26.055	85.909	37.34	30.012	20.745	24.965
Peak 12	15.369	0	4.297	0	0	0	0	4.184	59.607	12.017	715.156	17.486	0	0	0	0	0	0	0
Peak 13	16.769	268.464	254.287	332.222	223.974	297.253	276.262	4.987	80.869	105.596	101.655	37.909	16.52	4.036	6.395	2.302	2.93	3.776	3.455
Peak 14	24.38	10.506	19.292	22.663	14.36	19.949	0	47.644	72.169	105.354	87.41	75.017	84.614	0	0	0	0	0	0
Peak 15	25.591	313.298	248.279	307.402	253.594	335.299	127.654	933.234	1410.566	2802.648	743.652	1454.979	1688.599	53.765	12.931	19.444	44.482	52.963	33.33
Peak 16	27.037	206.781	261.014	304.775	165.495	203.816	232.503	35.836	25.73	112.87	0	133.575	83.814	7.068	6.979	4.04	4.824	8.346	5.313
Peak 17	28.156	0	0	0	0	0	0	12.316	42.778	4.016	45.635	0	4.57	0	0	0	0	0	0
Peak 18	29.046	0	0	0	0	0	9.295	8.351	57.106	0	63.64	0	12.355	0	0	0	0	0	0
Peak 19	38.773	8.901	19.671	18.232	10.053	15.269	10.211	39	36.889	86.138	66.39	39.183	68.284	0	0	0	0	0	0

total variation came from PC1 (51.2%) and PC2 (27.7%). Therefore, the multi-dimensional original data matrix was reduced to a two-dimensional dataset. As shown in Fig. 4, 18 samples in the PCA were found to be characteristically scattered in Regions 1, 2 and 3, with VA samples being in Region 1 (S1–S6), venison samples in Region 2 (S7–S12) and deer-bone samples in Region 3 (S13–S18) (Fig. 4). The PCA biplot is shown in Fig. 5. The 18 samples (S1–S18) were clearly divided into three groups, among which 10 peaks (Numbers 10, 13, 16, 8, 1, 2, 9, 14, 4 and 3) contributed greatly to this classification of samples. In the PLS-DA model, Q2 = 0. 934 (prediction ability of the model: the closer the Q2 value is to 1, the stronger the model prediction ability, which reflects the degree of separation of tissue samples), R2Y = 0. 966 (interpretation rate of the model: the closer the R2Y value is to 1, the closer the predicted value for each sample is to the real value for the sample), both of which are greater than 0.5, indicating that the model established in this experiment is reliable. VIP values of each peak from the samples were sorted (Fig. 6), and the larger the VIP value is, the greater the contribution of the peak to the sample classification. If a

Table 5. Similarity analysis of the 18 samples
Samples S1–S6 are from velvet antler (VA), Samples S7–S12 from venison, and Samples S13–S18 from deer bone

Sample	S1	S2	S3	S4	S5	S6
S1	1	0.891	0.952	0.984	0.981	0.893
S2	0.891	1	0.981	0.949	0.95	0.946
S3	0.952	0.981	1	0.987	0.983	0.957
S4	0.984	0.949	0.987	1	0.995	0.921
S5	0.981	0.95	0.983	0.995	1	0.936
S6	0.893	0.946	0.957	0.921	0.936	1
S7	0.587	0.786	0.725	0.692	0.715	0.672
S8	0.696	0.753	0.737	0.756	0.778	0.642
S9	0.706	0.561	0.605	0.689	0.706	0.469
S10	0.535	0.76	0.693	0.649	0.671	0.657
S11	0.708	0.791	0.762	0.77	0.796	0.68
S12	0.649	0.785	0.744	0.735	0.758	0.668
S13	0.76	0.669	0.693	0.753	0.776	0.59
S14	0.579	0.82	0.72	0.65	0.684	0.748
S15	0.67	0.817	0.777	0.748	0.783	0.76
S16	0.748	0.718	0.721	0.764	0.79	0.628
S17	0.755	0.642	0.669	0.734	0.759	0.574
S18	0.77	0.761	0.756	0.789	0.817	0.682

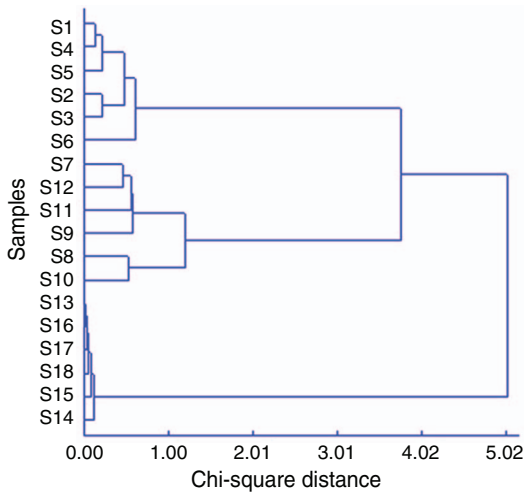

Fig. 3. Dendrogram of cluster analysis for the 18 samples from three types of deer tissue. Samples S1–S6 are from velvet antler (VA), Samples S7–S12 from venison and Samples S13–S18 from deer bone. Note that samples from each tissue type were grouped together; that is, three types of deer tissue have been unambiguously separated using cluster analysis.

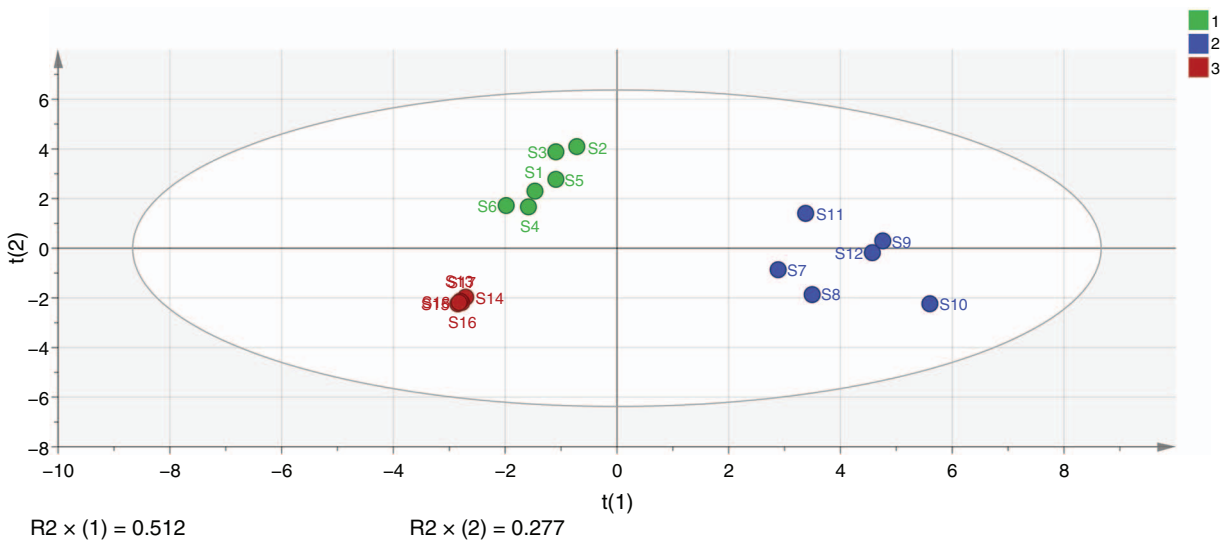

Fig. 4. Principal component-analysis (PCA) score plot. Each point in the figure represents a sample. Note that samples from each tissue type were characteristically located in different regions, namely, S1–S6 from velvet antler (VA) in Region 1, S7–S12 from venison in Region 2 and S13–S18 from deer bone in Region 3. The results suggest that PCA can also clearly separate the three types of deer tissue.

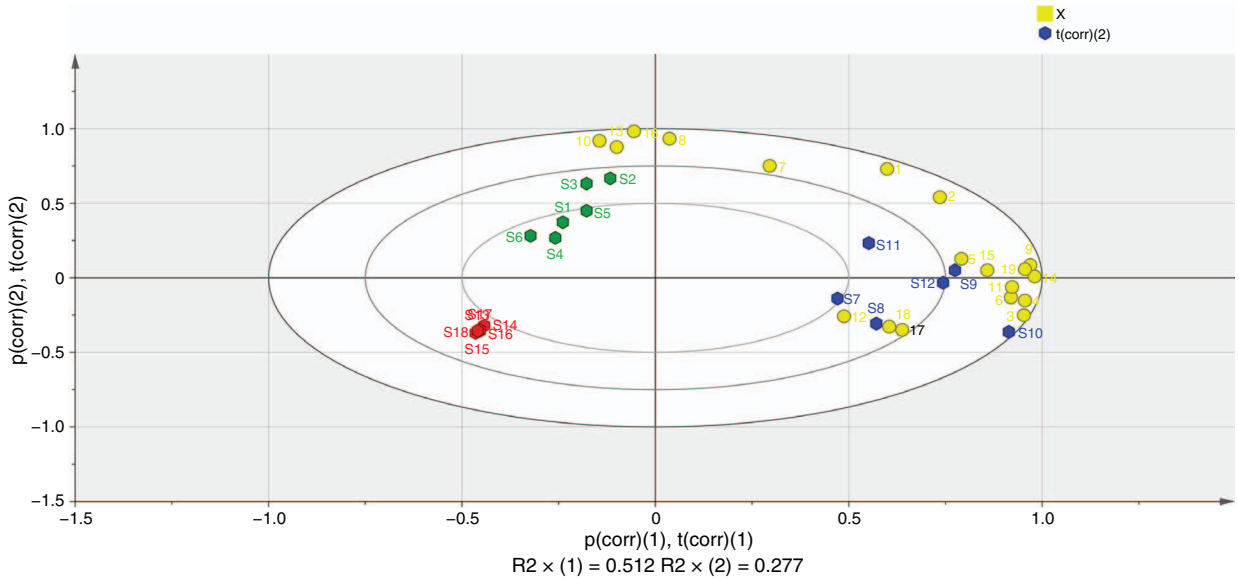

Fig. 5. Principal component-analysis (PCA) biplot. Biplot chart has integrated all the information of the PCA score chart and the load chart. We now can clearly see the separation among the different types of sample and the contribution of each peak to the grouping of samples. S1–S18 in the figure represent the 18 samples (PCA score plot), 1–19 are the common peaks obtained by similarity evaluation-system (SES) software from the 18 samples (PCA loading plot).

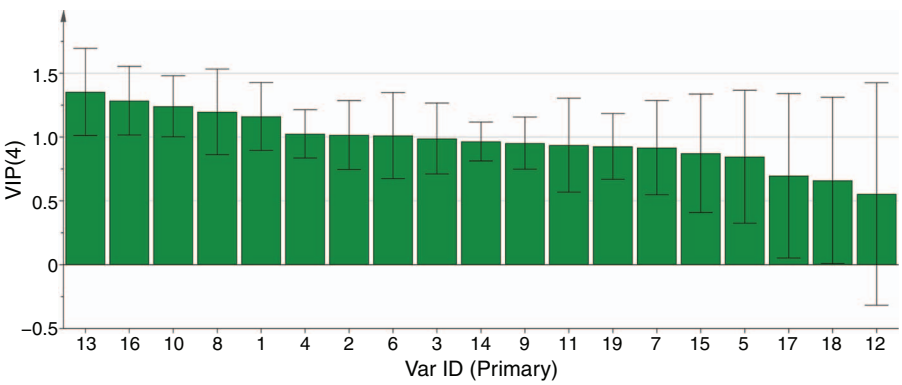

Fig. 6. Variable importance projection (VIP) values of each peak from each tissue sample. The larger the VIP value is, the greater the contribution of the peak to the sample classification. The VIP-value of >1 indicates that the peak is an important variable. Note that the VIP values of peaks Number 13, 16, 10, 8, 1, 4, 2 and 6 were all >1, indicating that these eight peaks have significant effects on separation of the three types of deer tissue.

VIP-value is >1, then the peak is an important variable. The results showed that the VIP values of peaks Number 13, 16, 10, 8, 1, 4, 2 and 6 were all >1, indicating that these eight peaks have significant effects on separation of the three types of deer tissue. Combined with the results of VIP plot and biplot, seven chromatographic peaks (Numbers 13, 16, 10, 8, 1, 2 and 4) were selected in the study for effectively distinguishing VA, venison and deer bone.

Discussion and conclusions

The HPLC fingerprints of three different types of deer tissue, namely, VA, venison and bone, were successfully established; HPLC method used in the present study was validated, having good precision, repeatable and stability. Our research found that VA lacked peaks Number 3, 4 and 17 compared with venison, but had six extra peaks, namely, Numbers 2, 5, 8, 9, 14 and 19, compared with deer bone. On the basis of the comparative results of HPLC fingerprints, there was significant difference in the number and area of peaks among VA, venison and deer bone. Furthermore, cluster analysis and PCA were performed on the18 samples, and were found to be consistent with each other. VA, venison and deer bone were completely separated and clustered into three categories using these analyses.

High-performance liquid-chromatography fingerprints of the three types of deer tissue were successfully established

for the first time in the present study. The established HPLC fingerprints were found to be able to comprehensively distinguish different types of deer tissue in combination with chemometric methods. We believe that our method can provide a feasible way for the quality control of VA, as well as other medicinal materials associated with deer in general, and support for the development of deep-processed VA products.

Conflicts of interest

The authors declare no conflicts of interest.

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