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ORIGINAL ARTICLE

Research on Mechanism of Traditional Hot Processing on Velvet Antler Based on ^{1}H Nuclear Magnetic Resonance and Multivariate Statistical Analysis

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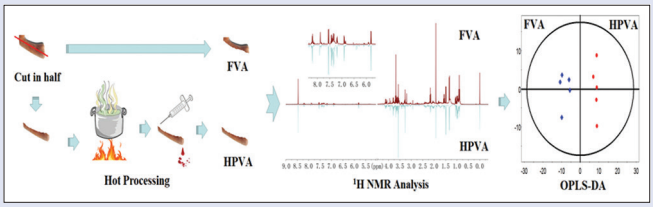
ABSTRACT

Background: The processing of Chinese materia medica is an important part for the preparation of traditional Chinese medicine (TCM) which has been believed that can change the chemical composition and physiological function. Velvet antler is a kind of precious TCM and widely used after hot processing as folk medicines in China. However, no strategy has been presented to reveal the processing principle of velvet antler. **Objective:** The aim of this study is to develop a method for exploring the mechanism of hot processing on velvet antler. **Materials and Methods:** In this study, the chemical compositions of fresh velvet antler (FVA) and hot processed velvet antler (HPVA) were compared based on the ^{1}H nuclear magnetic resonance (^{1}H NMR) and multivariate statistical analysis methods to explore the mechanism of hot processing on velvet antler. **Results:** It showed that hot processing increased phosphorylcholine, taurine, alanine, uridine, phenylalanine, uracil, tyrosine, accompanied by decreased bile acid, choline, lipid 2 and succinic acid in comparison to the FVA, which changed significantly in 52 common metabolites of FVA and HPVA. In addition, a clear separation between FVA and HPVA was obtained by using principal component analysis and 11 "chemical markers" were found by using orthogonal partial least squares discriminant analysis (OPLS-DA) approaches. Particular attention was given to the "chemical markers" with potentially beneficial bioactivities. **Conclusion:** The study demonstrated that ^{1}H NMR-based chemometric approach could be a promising tool for investigation of the hot processing of velvet antler in a holistic way of TCM.

Key words: ^{1}H nuclear magnetic resonance, hot processing, multivariate statistical analysis, traditional Chinese medicine, velvet antler

SUMMARY

- The chemical compositions of fresh velvet antler (FVA) and hot processed velvet antler (HPVA) were analyzed by ^{1}H nuclear magnetic resonance (^{1}H NMR)-based metabolomics approach for the first time
- 52 compounds were identified and 11 compounds were considered as potential "chemical markers" concerning FVA and HPVA
- ^{1}H NMR spectroscopy and chemometric tools are recommended as a holistic way of traditional Chinese medicine for studying the hot processing mechanism of velvet antler.



Abbreviations used: TCM: Traditional Chinese medicine; FVA: Fresh velvet antler; HPVA: Hot processed velvet antler; ^{1}H NMR: ^{1}H nuclear magnetic resonance; PCA: Principal component analysis; OPLS-DA: Orthogonal partial least squares discriminant analysis.

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INTRODUCTION

Velvet antler, the immature unossified horn of *Cervus nippon* Temminck or *Cervus elaphus* Linnaeus, a traditional Chinese medicine (TCM), has been recorded in the 2015 edition of the Chinese Pharmacopoeia,[1] which is believed to invigorate kidney yang, promote blood flow, and strengthen the bones and muscles. Modern pharmacological studies show that velvet antler exhibits wide functions, such as protection of myocardial injury, anti-aging, immune modulation, and facilitation of learning and memory.[2] Velvet antler has been reported to contain amino acids, proteins, peptides, polyamines, vitamins, hormones, nucleic acids, bases, lipids, aromatic compounds, and inorganic elements.[3]

Processing of medicinal materials is a traditional pharmaceutical technique used to fulfil the different requirements of storage, dispensing, and making preparations according to TCM theory. The aims of processing are to increase medical potency and reduce toxicity and side effects of crude drugs.[4,5] The growth inhibition rate of hot proceed antler hepatoma carcinoma cell was higher than fresh antler.[6] Processed velvet antler is widely used in the clinic in

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TCM for the treatment of invigorating kidney yang. In this work, the velvet antler metabolites of two differently processed products were compared.

As an animal medicine, the processing method of velvet antler has an important influence on its effective components and curative effect and directly relates to the quality of velvet antler. Conventionally, hot processed velvet antler (HPVA) was made by boiling fresh velvet antler (FVA) at 100°C for a short time and draining the residual blood, which have been repeated several times and then drying at 70°C. The purpose of hot processing is to make velvet antler dry and antiseptic, which is convenient for storage, transportation, and utilization.[7] For analyzing various compositions of the velvet antler well-established techniques currently used include the Folin-phenol method, the sodium dodecyl sulphate–polyacrylamide gel electrophoresis, reversed-phase high-performance liquid chromatography , and liquid chromatography-mass spectrometry.[8-11] Beyond these traditional methods, which are mostly investigated for the changes of a certain type components between FVA and HPVA, the ^{1}H nuclear magnetic resonance (^{1}H NMR) technique can be used to characterize the overall variation of a variety of water-soluble small molecules.

^{1}H NMR spectroscopy, which has an informative, nondestructive and nontargeted nature,[12] coupled with multivariate analyses such as principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) have been applied for the discrimination of different donkey-hide gelatin samples produced by different manufacturers.[13] Donkey-hide gelatin, like antler, is a kind of precious animal TCM. Thus, the metabolite profiling of FVA and HPVA could be investigated with the ^{1}H NMR method combined with multivariate analysis methods.

In the present study, a comparative analysis of the chemical constituents of the processed and unprocessed velvet antler was performed based on ^{1}H NMR spectra combined with PCA and OPLS-DA, to clarify the mechanism of the hot processing of velvet antler in accordance with the holism view of TCM. Furthermore, a reference point for a convenient method was provided for investigation of the processing of animal medicine in TCM.

MATERIALS AND METHODS

Chemicals and materials

The FVA of *Cervus nippon* Temminck was purchased from Dongfeng in Jilin Province of China. Chromatographic grade acetonitrile, analytical grade $Na_2HPO_4{\cdot}12H_2O$, and $NaH_2PO_4{\cdot}2H_2O$ were purchased from Beijing Chemical Works (Beijing, China). Sodium 3-trimethlysilyl [2, 2, 3, 3-d4] propionate (TSP) was acquired from Cambridge Isotope Laboratories Inc., (Andover, MA, USA).

Preparation of fresh velvet antler and hot processed velvet antler

The fresh sika deer antlers (two-branched) were burned the hair with alcohol lamp and cut into two portions in the longitudinal direction. One portion of FVA was wrapped with plastic wrap and boiled at 100°C for 1 min and then drained the residual blood, which have been repeated 5 times. Subsequently, that portion was dried at 70°C as HPVA sample, accompanied by using the other portion as FVA sample for the experiments. Then ten samples were gotten including five FVA samples and five HPVA samples after five sika deer antlers were proceed according to the above method.[14]

Sample preparation for ^{1}H nuclear magnetic resonance analysis

Twenty grams of FVA sample or HPVA sample was weighed and 200 mL of deionized water was added for homogenizing, followed by extraction conducted at 4°C for 24 h, the supernatant was collected by centrifugation at 3600 *r*/min for 10 min and freeze-dried. Forty milligrams of lyophilized powder was weighed into a 10 mL centrifuge tube and added to 5 mL 80% acetonitrile (v/v). The solution was dissolved by ultrasound for 1 min and centrifuged at 4000 *r*/min for 30 min and then, the solvent was evaporated at 75°C in a water bath. Dried extract was added to 500 μL buffer (pH 7.4 with Na_2HPO_4 and NaH_2PO_4, 0.1 M) and 100 μL D_2O (containing 0.05% TSP [m/v]). The solution (600 μL) was transferred into a 5-mm NMR tube for analysis.[15] All of 10 samples, including five FVA samples and five HPVA samples, were treated with the above method for ^{1}H NMR analysis.

Spectra acquisition and processing

^{1}H NMR experiment was recorded at 25°C on a Bruker AVANCE III NMR spectrometer (Bruker, Karlsruhe, Germany) operating at 500 MHz. ^{1}H NMR spectra used standard NOESYPRGP1D pulse sequence. Waiting time (3s) and mixing time (300 m) used lower powered continuous wave pulse for water peak suppression. With deuteroxide lock field, the internal standard is TSP. Each ^{1}H NMR spectrum consisted of 64 scans requiring 5-min acquisition time with the following parameters: 0.18 Hz/point, pulse width = 5498.53 Hz, at 90° (12.08 μs), sampling time 3.997s, and relaxation delay = 5.0 s.

Data analysis

The ^{1}H NMR spectrum was processed using Mest Re Nova (version 9.0.0, Mestrelab Research, Santiago de Compostella. Spain). Every spectrum was subsequently segmented at 0.04 ppm intervals across the chemical shift δ 0.00–9.00 ppm and the signal intensity in each region were integrated. The regions of δ 4.70–5.10 were excluded from the analysis because of the residual signal of HDO. SIMCA-P 11.0 (Umetrics, Umeå, Sweden) was used for PCA, projections to latent structures-discriminant analysis (PLS-DA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) of the NMR data to find differential metabolites.[16,17]

RESULTS AND DISCUSSION

Metabolites identification by ^{1}H nuclear magnetic resonance analysis

To illustrate the differences in metabolites between FVA and HPVA, 10 samples from five different velvet antlers were purchased and analyzed with the ^{1}H NMR spectroscopic technique. Identification of the metabolites in the two groups of velvet antler unveiled diversity in metabolite profiles.

Each compound is not only a chemical shift and couple constant but also most of the compounds are several sets of chemical shifts, split pattern, and couple constant that constitute a "three-dimensional data set" for the identification, which is based on the identification results obtained by referring to metabolomics literature. ^{1}H-NMR data and assignments of the metabolites of FVA and HPVA have the same peak profils including chemical shift, split pattern and couple constant of the common peaks, so only the spectrum of FVA is used as a representative to illustrate the identification of all metabolits in FVA and HPVA [Figure 1]. The metabolites were confirmed by comparing with the chemical shifts, split pattern and couple constant of standard

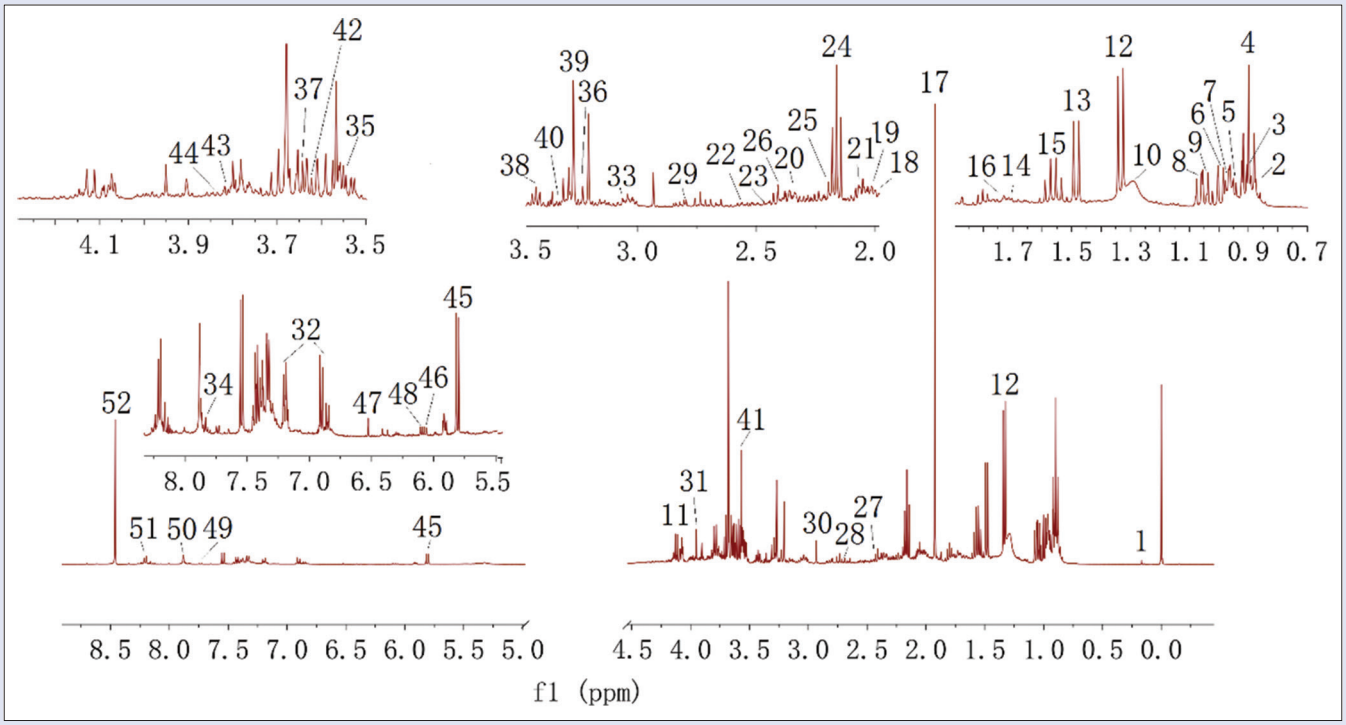

Figure 1: ^{1}H nuclear magnetic resonance spectra of fresh velvet antler

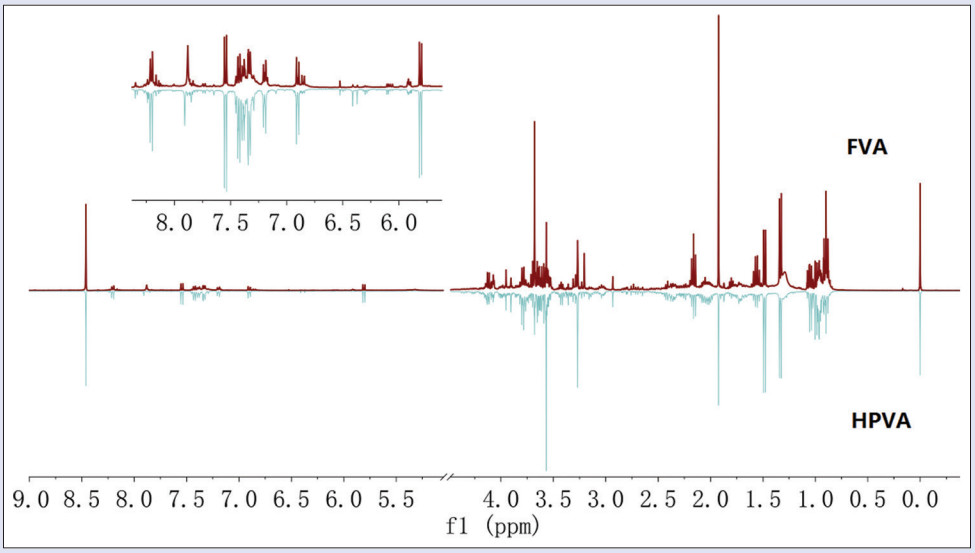

Figure 2: ^{1}H nuclear magnetic resonance comparison chart of fresh velvet antler and hot processed velvet antler

compounds from the Biological Magnetic Resonance Data Bank, Human Metabolome Database, as well as the literature [Table 1].[18-29] The main metabolites are amino acids, organic acids, choline, and nucleoside compounds, which are consistent with the literatures.[30-32] The results of the identification are shown in Table 1, and the comparison of FVA and HPVA is shown in Figure 2.

Multivariate statistical analysis of ^{1}H-NMR data

The ^{1}H NMR data were subjected to various multivariate data analyses to determine the chemical differences between FVA and HPVA.

Analysis of PCA is a commonly used unsupervised learning method in multi-dimensional statistical analysis.[33] In search of the principal metabolites, which emphasize the chemical changes in processing, a comparison of FVA and HPVA through PCA was conducted for the metabolomic study. Its scatter plot can directly indicate the difference between FVA and HPVA and it was clear that processed and unprocessed could be divided into two groups, indicating that the variety and/or content of the chemical components in HPVA had significantly changed [Figure 3]. Five HPVA samples were proceed by a series of complex treatments including heating, bleeding, and drying from five different FVA, so there were more differences within the HPVA group.

Table 1: ^{1}H nuclear magnetic resonance data of major metabolites in fresh velvet antler and hot processed velvet antler

Number	Name	δ/ppm	References
1	Bile acid	0.74 (s)	[18,19]
2	LDL	0.87 (m)	[18]
3	VLDL	0.89 (m)	[18]
4	Fatty acid	0.90 (t)	[17]
5	Pantothenate	0.94 (s), 0.90 (s)	[20]
6	Isoleucine	0.94 (t, J=6.6 Hz), 1.02 (d, J=7.2 Hz)	[18,21]
7	Leucine	0.96 (d, J=6.6 Hz), 0.97 (d, J=6.0 Hz)	[18,21]
8	Valine	0.99 (d, J=7.2 Hz), 1.05 (d, J=7.2 Hz), 2.27 (m)	[18,21]
9	3-OH-butyrate	1.02 (d, J=6.0 Hz), 2.31 (d), 2.41 (m)	[18]
10	Lipid 1	1.28 (m)	[18]
11	Lactate	1.33 (t, J=6.9 Hz), 4.12 (q, J=6.9 Hz)	[18,21]
12	Threonine	1.33 (d, J=6.6 Hz), 4.26 (m), 3.59 (d)	[22]
13	Alanine	1.48 (d, J=7.2 Hz), 3.77 (q, J=7.2 Hz)	[18,19]
14	Lysine	1.53 (m), 1.72 (m), 1.90 (m), 3.03 (t, J=9.0 Hz), 3.77 (t, J=7.2 Hz)	[23]
15	Lipid 2	1.58 (m)	[18]
16	Arginine	1.73 (m), 1.93 (m), 3.24 (t, J=7.2)	[24]
17	Acetic acid	1.92 (s)	[18,21]
18	Proline	2.01 (m), 2.07 (m), 2.36 (m), 3.34 (m), 3.45 (m), 4.13 (m)	[25]
19	Pyroglutamate	2.04 (m), 2.42 (m), 2.52 (m), 4.18 (q)	[26]
20	Glutamate	2.06 (m), 2.35 (m)	[18,21]
21	Glutamate ester	2.10 (m), 2.09 (m), 2.36 (m), 3.77 (m)	[27]
22	Methionine	2.14 (s), 2.64 (t, J=7.8 Hz), 3.85 (m)	[18]
23	Glutamine	2.15 (m), 2.44 (m), 3.77 (m)	[18,19]
24	Oxidized glutathione	2.16 (m), 2.54 (m), 2.97 (dd), 3.31 (dd), 4.76 (t)	[18]
25	Acetoacetate	2.27 (s)	[22]
26	Pyruvate	2.38 (s)	[22]
27	Succinic acid	2.41 (s)	[25]
28	Citric acid	2.53 (d, J=16.2 Hz)	[21]
29	Aspartate	2.68 (m), 2.82 (m)	[21,28]
30	Dimethylglycine	2.93 (s)	[22]
31	Creatinine	3.04 (s), 3.94 (s)	[25]
32	Tyrosine	3.06 (dd), 3.15 (dd), 3.94 (dd), 6.91 (d, J=8.4 Hz), 7.20 (d, J=8.4 Hz)	[28]
33	Phenylalanine	3.13 (dd), 3.29 (dd), 3.98 (dd), 7.33 (m), 7.43 (m)	[18]
34	Histidine	3.14 (dd), 3.25 (dd), 3.99 (dd), 7.83 (s)	[19]
35	Choline	3.22 (s), 3.52 (t, J=5.4 Hz), 4.07 (t)	[29]
36	Phosphorylcholine	3.22 (s), 3.61 (t), 4.19 (t)	[25]
37	Glycerophosphocholine	3.23 (s), 3.68 (t), 4.32 (t)	[27]
38	Taurine	3.25 (t), 3.43 (t)	[27]
39	Betaine	3.27 (s), 3.91 (s)	[22]
40	Tryptophan	3.31 (dd), 3.49 (dd), 4.06 (dd), 7.21 (t), 7.29 (t), 7.33 (d), 7.55 (d), 7.74 (d)	[28]
41	Glycine	3.56 (s)	[28]
42	Myo-inositol	3.62 (s)	[28]
43	Uridine	3.81 (d), 3.92 (d), 4.14 (q), 4.24 (t), 4.36 (t), 5.90 (d), 5.91 (d), 7.87 (d)	[22]
44	Inosine	3.85 (dd), 3.92 (dd), 4.28 (q), 4.44 (t), 6.10 (d), 8.24 (s), 8.34 (s)	[23]
45	Uracil	5.81 (d, J=9.0 Hz), 7.55 (d, J=9.0 Hz)	[22]
46	Cytidine	6.08 (d), 7.85 (d)	[23]
47	Fumaric acid	6.53 (s)	[18]
48	Adenosine	6.10 (d, J=7.2 Hz), 8.24 (s), 8.35 (s)	[25]
49	Niacinamide	7.66 (dd), 8.72 (d)	[18]
50	Xanthine	7.88 (s)	[18]
51	Hypoxanthine	8.20 (s), 8.22 (s)	[18]
52	Formic acid	8.44 (s)	[18,25]

The assignment of peaks was visually assigned in Figure 1. LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein; s: Singlet; d: Doublet; t: Triplet; q: Quartet; m: Multiplet; dd: Doublet of doublets

PLS-DA is a supervised discriminant technique to process large data set and can reduce the differences of inter-group to the lowest level. In general, the fitness of model was indicated by R^2 and the predictive ability was represented by Q^2.[14] In order to improve the separation between FVA and HPVA groups and interpret the potential discriminant metabolites of the two groups, OPLS-DA is performed and validated by the corresponding PLS-DA models. It showed that the model had excellent predictive abilities, which $R^2 = 0.637$ and $Q^2 = -0.36$ were obtained [Figure 4a].[34]

A clear separation in the OPLS-DA score plot between FVA and HPVA groups was acquired, which revealed that they had different metabolic profiling [Figure 4b]. From the S-score plot constructed from OPLS-DA [Figure 4c], metabolites which had significant differences between FVA and HPVA groups could be found. In this study, 11 significant differences metabolites, as "chemical markers" for discriminating between FVA and HPVA, were selected based on their distance from the origin in the S-plot, variable importance in projection values larger than 1.0 and $P < 0.05$ [Table 2].

Comparison of metabolite content between fresh velvet antler and hot processed velvet antler

Taking TSP as the internal standard, the 15 components of the identified compounds with obvious characteristic peaks without overlapped peaks with other substances were manually integrated [Table 2]. The differences in the content of 11 metabolites had the significance of statistics ($P < 0.01$) by using of *t*-test with SPSS version 16.0 (SPSS Inc., Chicago, IL). Levels of fatty acid, phosphoryl-choline, taurine, alanine, uridine, phenylalanine, uracil, and tyrosine in HPVA were significantly ($P < 0.01$) higher than in the samples from FVA, on the contrary, the contents of choline, lipid 2, and succinic acid were higher in FVA than in HPVA.

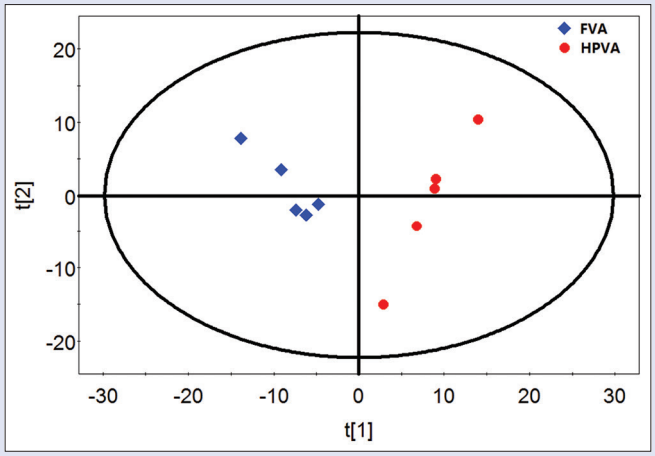

Figure 3: Principal component analysis score plots of fresh velvet antler and hot processed velvet antler, fresh velvet antler (♦), hot processed velvet antler (●)

The results were basically consistent with the multivariate statistical analysis, which 11 extremely significant differences components are illustrated in the S-plots [Figure 4c].

The most significant components to discriminate FVA and HPVA are amino acids, organic acids, and nucleosides. The contents of alanine, phenylalanine, and tyrosine were higher in HPVA, which were hydrophobic amino acids. This may be due to the breaking of peptide bonds of protein and polypeptide contained in FVA by heat treatment, which produced more free hydrophobic amino acids. The decrease of the content of hydrophilic threonine may be due to dissolving in hot water during boiling and discharging blood. In addition, the content of taurine, uridine, and uracil was increased in HPVA. Uridine, uracil, and succinic acid had a certain immune-enhancing activity.[35,36] Moreover, taurine, high concentration of saturated fatty acid could significantly promote the secretion of testosterone in the rat Leydig cells *in vitro*,[37,38] which may be caused by taurine involved in the biosynthesis pathway of steroid hormone. Kidney-yang deficiency is related to the neuroendocrine-immune system in modern medicine including enhancing immune function and regulating sex hormone content.[39] It is suggested that the change in the content of the above "chemical markers" is one of the reasons for the invigorating Kidney Yang activity of HPVA.

CONCLUSION

Hot processing of velvet antler is a traditional method, and it has great significance for the efficacy of antler. This is the first time that a metabolic profiling analysis, using ^{1}H NMR and multivariate data analysis, was applied to explain the processing mechanism of antler. The results presented here suggested that HPVA differed from FVA greatly on the chemical composition. The information obtained in this work is valuable for identifying fresh or processed products of velvet antler and establishing the analytical requirements for quality control in future. It is of practical significance for the modern research of velvet antler and to ensure the safety and efficacy of clinical medicine. In addition, further research of their respective bioactivity

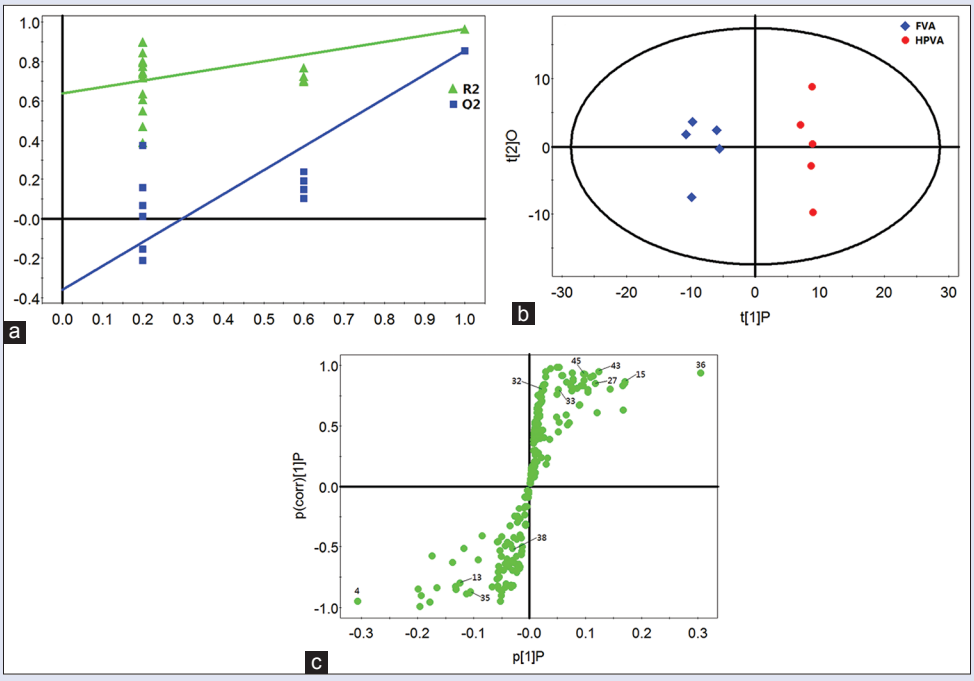

Figure 4: Projections to latent structures-discriminant analysis model (a), score plot (b) and S-plot (c) of Orthogonal projections to latent structures-discriminant analysis results obtained from ^{1}H nuclear magnetic resonance spectra of the different samples: Fresh velvet antler (♦) and hot processed velvet antler (●)

Table 2: The trend of relative content of hot processed velvet antler

Number	Name	δ/ppm	VIP	Trend
4	Fatty acid	0.9 (t)	4.54797	↓**
41	Glycine	3.56 (s)	4.45679	↑
35	Choline	4.07 (t)	2.97965	↓**
36	Phosphorylcholine	3.61 (t)	2.97965	↑**
8	Valine	0.99 (d, J=7.2Hz), 1.05 (d, J=7.2 Hz)	2.42302	↑
7	Leucine	0.96 (d, J=6.6 Hz), 0.97 (d, J=6.0 Hz)	2.42302	↑
38	Taurine	3.25 (t), 3.43 (t)	2.33114	↑**
13	Alanine	1.48 (d, J=7.2 Hz)	2.10182	↑**
12	Threonine	1.33 (d, J=6.6 Hz)	1.99317	↓
15	Lipid 2	1.58 (m)	1.93861	↓**
43	Uridine	3.81 (d)	1.84108	↑**
33	Phenylalanine	7.43 (m)	1.51588	↑**
45	Uracil	5.81 (d, J=9.0 Hz), 7.55 (d, J=9.0 Hz)	1.43256	↑**
27	Succinic acid	2.41 (s)	1.17198	↓**
32	Tyrosine	6.91 (d, J=8.4 Hz)	1.07951	↑**

*P<0.05; **P<0.01 versus FVA. ↑: Higher in HPVA; ↓: Lower in HPVA; HPVA: Hot processed velvet antler; s: Singlet; d: Doublet t; t: Triplet; m, Multiplet; FVA: Fresh velvet antler; VIP: Variable importance in projection

is also important and could be conducted to fully understand the essence of hot processing, which be linked to clinical practice.

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Conflicts of interest

There are no conflicts of interest.

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