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ORIGINAL ARTICLE

A multi-evaluating strategy for raw and processed *Veratrum nigrum* L.: Fingerprinting combined with quantitative analysis based on multivariate chemometric methods

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Abstract *Veratrum nigrum* L. (VN) is a well-known herbal medicine and rich in chemical components with multiple pharmacological activities including antihypertensive, anticancer, and antifungal effects. In the current experiment, the quality of VN from different habitats was evaluated based on combinative method of fingerprint, multi-component quantification and chemical pattern recognition. Fifteen batches of VN were collected, and intrinsic chemical composition were identified using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry, which is a method for analyzing the similarity between samples, coupled with fingerprint of traditional Chinese medicine. The fingerprint similarity model show that 22 common peaks were selected covering 15 batches of and the similarity > 0.963. The total of 22 joint components were tentatively identified by comparison with standard substances or literature. A ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry method for simultaneous determination of 8 compounds was established to evaluate the contents of raw and processed *Veratrum nigrum* L. Multivariate analysis was then applied to compare different batches of herbs based on ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry data. All raw and processed samples were classified by partial least squares dis-

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criminant analysis based on the 8 analyzed compounds. The findings suggested that veratramine and polydatin with a variable importance for the project (VIP) > 1 were identified as significant constituents, the presence of which can be used to differentiate between raw and processed *Veratrum nigrum* L. samples. These results indicate that processing methods show important effects on the composition of *Veratrum nigrum* L..

1. Introduction

Veratrum nigrum L., named as "LiLu", originate from the dry roots and rhizomes of plants in the *Liliaceae* family (Fan et al., 2020). It has been widely used in traditional medical systems in China, due to their reliable therapeutic efficacy. The components of VN are alkaloids, flavonoids and stilbene (Cong et al., 2008; Zheng, et al., 2019). Pharmacological and chemical studies have been revealed that the major therapeutic compounds of this herbal medicine are steroidal alkaloids (Szeliga et al., 2019). With internally use, VN treats bloodstroke, epilepsy and resolving phlegm, while with external use, it cures scabies, tinea capitis, and maggot killing (Kang, et al., 2015; Park, et al., 2016; Li et al., 2020).

Recently, the chemical composition and pharmacological action of VN has been widely reported, but its use has limitations due to its toxicity. In traditional Chinese medicines (TCMs) theory, processing (plain-frying or stir-frying until brown, steaming, braising, or stir-frying with wine) is necessary. It can promote its therapeutic effects and/or reduce side-effects of herbs by decreasing their levels of toxic constituents (Wang et al., 2017; Zhou et al., 2015). At present, the researches focusing on pharmacological activities show that processing method is a significant factor influencing its compositional contents (Chen et al., 2020; Zhang et al., 2016). It is worth noting that processed VN plays an important role in reducing the toxicity and promoting to be widely used in further clinical applications.

Because of the complexity, similarity and diversity of the chemical structures, the analysis of VN is still a great challenge. Fortunately, the fingerprint can describe integral characterization of VN and reflect the internal chemical composition information (Chen et al., 2018; Viapiana et al., 2016). UPLC-Q-TOF/MS can provide a high mass resolution and fragment ion, which will be the beneficial for structure elucidation and can be used in the identification of common peaks in fingerprint (Kadokami et al., 2019; Park et al., 2020; Gampe et al., 2016; Zhao et al., 2015). Meanwhile, Ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UPLC-MS/MS) is a powerful platform in the quantification of the main fingerprint components depending on its high separation capacity and sensitivity (Jiao, et al., 2019; Lin et al., 2019; Chan et al., 2019). Thus, the use of fingerprint in combination with Liquid chromatography-mass spectrometry (LC-MS) might exhibit the unique advantage for the analysis the discrimination of *Veratrum nigrum* L. samples.

In this study, fingerprint analysis and UPLC-Q-TOF/MS were used to identify the key chemical markers that are responsible for the characterization. Furthermore, a UPLC-MS/MS method was established to simultaneously determine 8 compounds including apigenin, cyclopamine, jervine, polydatin, quercetin, resveratrol, veratramine, and veratrosine within 5 min in VN. Moreover, the present assay further analyzes the main components of raw and processed VN using chemical pattern recognition. And it highlights the infuence of processing on the compounds and this information will be useful in the evaluation and diferentiation of raw and processed VN herbs.

2. Materials and methods

2.1. Materials, chemicals and reagents

Fifteen batches of raw VN were collected from different provinces in China and identified by Prof. Tianxiang Li from School of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine. Sample information is provided in Table 1. The voucher specimens were deposited at the Tianjin University of Traditional Chinese Medicine, China.

Acetonitrile and methanol (Merck & Co Inc, USA) were of HPLC grade. Formic acid of HPLC-grade was obtained from ROE Co., ltd. Deionized water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA). Apigenin, cyclopamine, jervine, polydatin, quercetin, resveratrol, and veratramine were purchased from Chengdu Desite Bio-Technology Co., ltd (Chengdu, China). Veratrosine was purchased from Chengdu Chroma-Biotechnology Co., ltd (Chengdu, China).

2.2. Processing methods of Veratrum nigrum L.

The roots and rhizomes of the plants were cut into segments, and were stir-fried in a metallic pan at 120–140 °C, over a medium flame for about 15 min, the color on the surface of samples turns brown and scorched spots appeared.

2.3. Preparation of standard solutions

Apigenin, cyclopamine, jervine, polydatin, quercetin, resveratrol, veratramine and veratrosine were accurately weighed and dissolved in methanol at a final concentration of 100 μg/mL as stock solutions, respectively. Working standard solutions were further obtained by diluting the above stock solutions in appropriate amounts.

2.4. Preparation of sample solutions

The roots and rhizomes of the plants were ground into powder form in an electric grinder and then passed through a 24-mesh (0.8 mm) sieve. Each sample was accurately weighed (10.0 g) and extracted 3 times by hydrodistillation with 150 mL of 70 % ethanol (v/v) for 2 h at 100 Volt. The supernate was filtered through a 0.22 μm filter membrane and was stored at 4 °C until analysis.

Table 1 Detailed information of *Veratrum nigrum* L. from 15 regions.

Batch	Region	Lot Number	Batch	Region	Lot Number	Batch	Region	Lot Number
1	Yunnan	20190917	6	Shanxi	20190620	11	Hubei	20190918
2	Jiangxi	20190920	7	Jilin	20190812	12	Heilongjiang	20190808
3	Fujian	20190817	8	Xinjiang	20190908	13	Guangdong	20190911
4	Shanxi	20190915	9	Guizhou	20190816	14	Zhejiang	20190813
5	Anhui	20190907	10	Guangxi	20190706	15	Anhui	20190826

2.5. Chromatographic and mass spectrometric conditions

2.5.1. UPLC-Q-TOF/MS acquisition analysis of fingerprint

UPLC-Q-TOF/MS system was composed of Agilent 1290 UPLC instrument (Agilent Technologies, Waldbronn, Germany) and Agilent 6520 Q-TOF mass spectrometer (Agilent Corporation, Santa Clara, CA, USA). The mass spectra data was acquired in the negative electrospray ion (ESI) mode. The chromatographic peaks were separated on an CORTECS® UPLC® C18 column (2.1 × 100 mm, 1.6 μm, Waters) at a flow rate of 0.3 mL/min. Mobile phase consisted of 0.1 % formic acid–water (A) and acetonitrile (B). The gradient elution program was set as: 0–5 min, 5 %–13 % B; 5–18 min, 13 %–21 % B; 18–20 min, 21 %–60 % B; 20–25 min, 60 %–62.5 % B; 25–34 min, 62.5 %–73 % B; 34–35 min, 73 %–76 % B. The post run time was 5 min. The injection was 1 μL. The related Q-TOF/MS parameters were listed as follows: drying gas, N_2; gas flow rate, 11 L/min; drying gas temperature, 350 °C; nebulizer gas pressure, 40 psi; capillary voltage, 3500 V; fragmentor voltage, 135 V; collision energy (CE), 30 V. The scan range of mass spectra was m/z 100–1700.

2.5.2. UPLC-MS/MS analysis

UPLC-MS/MS system consists of an Agilent 1290 ultra-high performance liquid chromatography system (Agilent Technologies, Germany) coupled with an Agilent 6470 series triple quadrupole mass spectrometer (Agilent Technologies, Singapore) with an electrospray ionization (ESI) source. The quantitative analysis was performed on a CORTECS® UPLC® C_{18} column (2.1 × 100 mm, 1.6 μm, Waters) at 20 °C with a flow rate of 0.3 mL/in and the injection volume of 5 μL. Mobile phase was a mixture of 0.1 % formic acid–water (A) and acetonitrile (B). The gradient program of mobile phase was as follows: 0–2 min, 15–47 % B; 2–5 min, 47–47 % B. Data analysis was performed using Masshunter Workstation Software from Agilent Technology (version B.09.00).

The multiple reaction monitoring (MRM) mode was applied for quantification in positive and negative ionization mode simultaneously. The optimum MS values were maintained as follows: nebulizing gas pressure, 35 psi; drying gas (N_2) flow rate, 7 L/min with a temperature at 300 °C; sheath gas flow, 11 L/min with a temperature at 350 °C. The precursor-product ion pairs used for the MRM detection and MS parameters are showed in Table 2.

2.6. Method validation

2.6.1. UPLC-Q-TOF/MS acquisition method validation

The precision, repeatability, and stability were investigated to validate the applicability of UPLC-Q-TOF/MS method by using the samples. For precision, the method was evaluated by intra-day variability. Relative standard deviations (RSDs) were calculated as the measure of precision. In the repeatability examination, six replicates of the samples from the same batch were extracted and analyzed. To evaluate the stability of the analytes, sample solutions were stored at room temperature and then analyzed by replicate injections at 0, 2, 4, 8, 10, and 12 h. RSDs were used to assess stability.

2.6.2. UPLC-MS/MS method validation

The linearity of the assay for the test compounds was assessed by least-square linear regression of the analyte-to-standard peak area ratio (y) versus the normalized standard concentration (x). Lower limit of quantification (LLOQ) for each sample was defined based on the concentrations that generated peaks with signal-to-noise values (S/N) of 10. The raw VN (batch 1) was applied to validate the method, including precision, repeatability, stability and recovery. For precision, the method was assessed by intra-day and inter-day variability. In the repeatability examination, six replicates of the samples from the raw VN (batch 1) were extracted and analyzed. The recovery was carried out by adding known amount of 8 standards solution to raw VN samples (5 g), which was used to further investigate the accuracy of the method. The samples were prepared according to the procedure described for sample preparation in section "2.4". Recovery was calculated using the following formula: recovery (%) = (amount found - original amount)/amount spiked × 100 %. To assess the stability of analytes, sample solutions were stored at room temperature and then analyzed by replicate injection at 0, 2, 4, 8, 10 and 12 h.

2.7. Statistical analysis

The original data of VN in multiple reaction monitoring modes were processed by the MassHunter software and Qualitative Navigator software. The chromatogram data (TXT format) from the UPLC-Q-TOF/MS system was imported into the "Similarity Evaluation System of Traditional Chinese Medicine Chromatographic Fingerprint" software (2004 edition, developed by the Chinese Pharmacopoeia Commission). Multivariate chemometric methods, such as hierarchical cluster analysis (HCA), principal component analysis (PCA) and partial least-squares discrimination analysis (PLS-DA), were applied to classify the new matrix data and determine their similarities and differences. Combined with chemical pattern recognition methods to synthesize the resulting fingerprint profiles, the results were judged to visually reflect the quality differences between samples of different origins. Statistical analyses were carried out using SPSS 25.0 and SIMCA-P 14.1 software.

Table 2 Mass spectra properties of 8 analytes.

Compound	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Fragmentor (V)	Collision Energy (V)	Ion mode
Veratrosine	572.4	457.2	242	40	Positive
Jervine	426.3	114.0	214	36	Positive
Cyclopamine	412.3	114.0	80	36	Positive
Veratramine	410.3	295.1	189	32	Positive
Polydatin	389.1	227.0	156	28	Negative
Quercetin	301.0	151.0	128	24	Negative
Apigenin	269.0	117.0	141	40	Negative
Resveratrol	229.1	107.0	93	24	Positive

The external standard method was used for accurate quantification of VN samples, and all chromatographic data obtained from UPLC-MS/MS was processed by the SIMCA-P software. Furthermore, PLS-DA, a supervised multivariate data analysis technique, characterized by variable selection criteria and potential biomarker output. The VIP > 1.0 was used as the criterion to further screen out the components that contribute more to the above samples classification. According to the VIP, potential biomarker were considered to be the significantly markers which could distinguish the differences of VN from different areas and processing procedures.

3. Results and discussion

3.1. Fingerprint analysis using UPLC-Q-TOF/MS

3.1.1. Method validation for fingerprint analysis

The retention times (Rt) and peak areas of 22 common compounds(*P*1-P22) were employed to calculate the RSD values, which were regarded as the important assessment indicator of precision, repeatability, and stability. The RSD values of precisions were all below 6.7 %, which displayed a high accuracy of Rt and peak areas of target ions in the process of multiple samples analysis by the UPLC-Q-TOF/MS method. Moreover, the repeatability with the RSDs ranging from 0.6 % to 6.9 % showed good consistency of results detected by UPLC-Q-TOF/MS. Finally, the RSDs indicative of stability were within 2.1 %-6.2 %, demonstrating that sample solutions were stable for qualitative detection in 12 h. All data are displayed in Table 3. These results indicated that UPLC-Q-TOF/MS method was applicable and reliable for acquiring the fingerprint data.

3.1.2. Similarity assessment of fingerprint

To achieve higher resolution, faster separation, and provide good responses to all chemical components in MS analysis, the UPLC chromatographic conditions and the MS conditions were optimized. Multiple reaction monitoring modes were employed to obtain as many corresponding MS and MS^2 signals as possible. Fifteen batches (S1–15) of VN were analyzed, and chromatograms were recorded. The chromatographic fingerprint of 15 batches of samples were shown in Fig. 1. Then, the chromatogram data (TXT format) from the UPLC-Q-TOF/MS system were imported into the "Similarity Evaluation System of Traditional Chinese Medicine Chromatographic Fingerprint" software (2004 edition, developed by the Chinese Pharmacopoeia Commission), as recommended by the China Pharmacopoeia Committee. The chromatogram of S1 was set as the reference spectrum, and a total of 22 common peaks (*P*1-P22) were aligned and marked manually. Moreover, 22 of common components were unambiguously identified by comparing their retention times, accurate masses with data from the corresponding standard substances, and comparison with the data from previous joint literature

Table 3 Precision, stability, repeatability of the RSD values for 22 common fingerprint peaks.

Compounds	Precision RSD (%)	Repeatability RSD (%)	Stability RSD (%)	Compounds	Precision RSD (%)	Repeatability RSD (%)	Stability RSD (%)
1	2.7 %	2.7 %	6.2 %	12	3.8 %	3.6 %	3.1 %
2	2.9 %	2.6 %	3.8 %	13	5.0 %	6.9 %	5.9 %
3	1.7 %	2.6 %	4.7 %	14	6.1 %	5.7 %	5.8 %
4	0.9 %	0.6 %	2.1 %	15	4.6 %	4.9 %	3.8 %
5	2.1 %	1.7 %	3.6 %	16	3.2 %	4.9 %	3.8 %
6	1.0 %	1.0 %	2.1 %	17	1.5 %	6.3 %	5.1 %
7	0.7 %	0.9 %	2.4 %	18	5.4 %	6.0 %	4.7 %
8	1.9 %	1.6 %	2.9 %	19	6.7 %	6.1 %	5.0 %
9	2.4 %	1.8 %	2.5 %	20	5.5 %	2.9 %	5.6 %
10	1.2 %	1.3 %	2.9 %	21	6.2 %	4.7 %	4.5 %
11	0.6 %	1.6 %	2.4 %	22	4.6 %	5.8 %	3.5 %

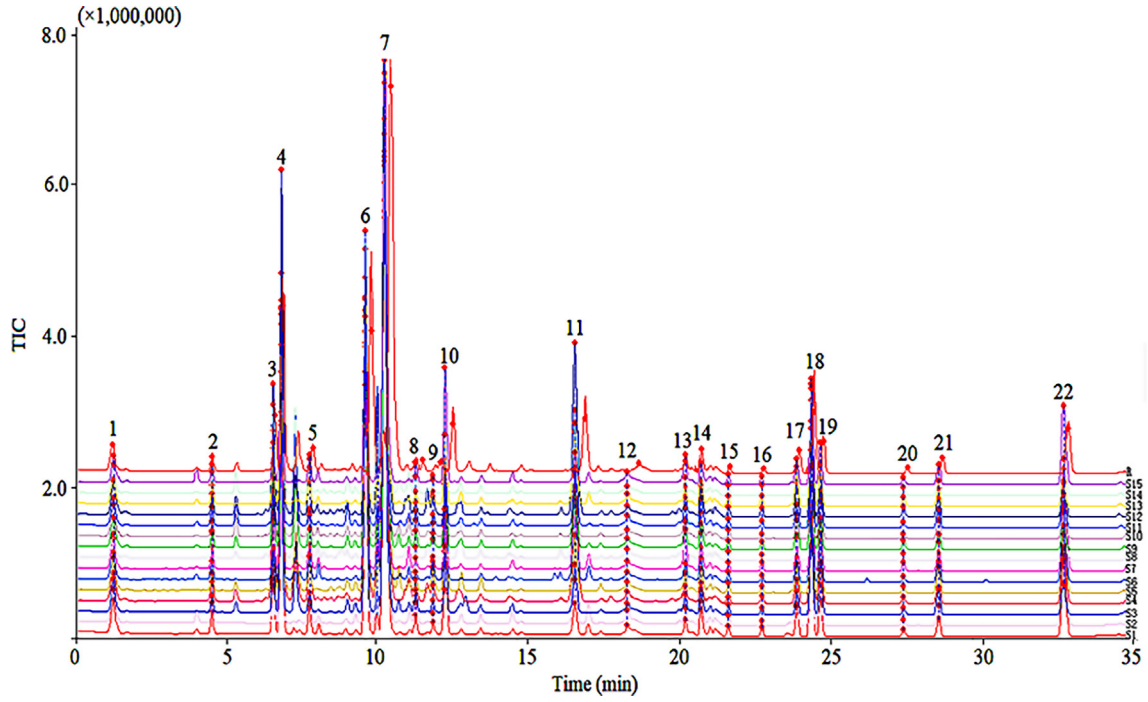

Fig. 1 Fingerprint of 15 batches of *Veratrum nigrum* L. (VN) were analyzed by the Similarity Evaluation System.

Table 4 Identification of 22 common components in fingerprints by UPLC-Q-TOF/MS.

No.	Rt (min)	*m/z*	Fragment Ion	Loading Form	Possible Compound	Molecular Formula	Diff (ppm)
1	1.24	104.0598	57.0348	$[M + H]^+$	2,3-diaminopropanoate	$C_3H_7N_2N_2$	1.05
2	4.50	460.3089	141.9587	$[M + H]^+$	Verdine	$C_{27}H_{41}NO_5$	−0.55
3	6.56	588.3315	508.5408, 536.3210	$[M + H]^+$	Pseudojervine	$C_{33}H_{49}NO_8$	−2.53
4	6.83	572.3629	410.3055, 536.5753	$[M + H]^+$	Veratrosine	$C_{33}H_{49}NO_7$	4.75
5	7.77	444.3131	426.2079	$[M + H]^+$	Vibeissine	$C_{27}H_{41}NO_4$	3.26
6	9.63	426.3018	102.1279	$[M + H]^+$	Jervine	$C_{27}H_{39}NO_3$	−3.54
7	10.26	410.3077	217.1029, 295.2057	$[M + H]^+$	Veratramine	$C_{27}H_{39}NO_2$	3.83
8	11.31	658.3615	640.3504	$[M + H]^+$	Veratroylzygadenine	$C_{36}H_{51}NO_{10}$	0.36
9	11.89	412.3233	125.9862, 110.0085	$[M + H]^+$	Cyclopamine	$C_{27}H_{41}NO_2$	2.14
10	12.29	576.3567	412.3215	$[M + NH_4]^+$	Cucurbitacin B	$C_{32}H_{46}O_8$	−2.74
11	16.62	398.3435	394.311	$[M + H]^+$	Solanidine	$C_{27}H_{43}NO$	1.11
12	18.39	228.1267	61.9271	$[M + H]^+$	Harmaline hydrochloride	$C_{14}H_{15}N_2O$	−4.35
13	20.28	343.2111	240.1603	$[M + H]^+$	Diethyllachesine	$C_{21}H_{28}NO_3$	3.1
14	20.80	219.1607	239.4640, 119.0312	$[M + H]^+$	Nitrocyanamide	$C_5H_{12}N_7O_3$	0.8
15	21.73	279.0825	121.064	$[M + H]^+$	Dimethyl 4,4-dinitroheptanedioate	$C_9H_{14}N_2O_8$	−0.88
16	22.84	279.1548	121.1477, 106.0173	$[M + NH_4]^+$	Lotaustralin	$C_{11}H_{19}NO_6$	0.78
17	24.00	399.2165	101.5266	$[M + H]^+$	Galbanic acid	$C_{24}H_{30}O_5$	0.35
18	24.48	282.2022	275.0844, 109.0520	$[M + H]^+$	7,7-diethoxyheptane-1,4-diamine	$C_{11}H_{27}N_3O_5$	0.57
19	24.80	281.1701	554.2748	$[M + H]^+$	(2,6-dimorpholin-4-ylpyrimidin-4-yl) hydrazine	$C_{12}H_{20}N_6O_2$	2.25
20	27.57	284.2151	146.0256	$[M + H]^+$	Bis (4-*tert*-butylphenyl) oxidanium	$C_{20}H_{27}O$	−5.88
21	28.73	256.1905	104.0621	$[M + H]^+$	C10-homoserine lactone	$C_{14}H_{25}NO_3$	0.2
22	32.89	360.2383	100.9134, 226.8619	$[M + H]^+$	Broussonetine H	$C_{18}H_{33}NO_6$	3.08

(Table 4). Most of the samples had a similarity level of 0.963–0.994 (Table 5), compared with the reference fingerprint (R). The above results showed that the compositions of 15 batches of VN from different area were the same.

3.1.3. Chemometric analysis of fingerprint

HCA, PCA and PLS-DA multivariate statistical methods were employed to further explore the difference in quality of different samples (Kandasamy et al., 2020; Zhang et al., 2021). HCA

Table 5 Similarities of 15 batches of *Veratrum nigrum* L. samples.

Batch	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	R
S1	1															
S2	0.989	1														
S3	0.978	0.968	1													
S4	0.936	0.907	0.953	1												
S5	0.987	0.983	0.992	0.94	1											
S6	0.978	0.971	0.98	0.947	0.975	1										
S7	0.984	0.986	0.972	0.933	0.973	0.984	1									
S8	0.951	0.945	0.971	0.975	0.965	0.97	0.966	1								
S9	0.979	0.962	0.996	0.958	0.988	0.981	0.974	0.972	1							
S10	0.967	0.948	0.99	0.978	0.981	0.975	0.959	0.98	0.988	1						
S11	0.958	0.953	0.989	0.931	0.974	0.97	0.965	0.959	0.988	0.974	1					
S12	0.931	0.914	0.951	0.985	0.942	0.95	0.944	0.992	0.957	0.972	0.939	1				
S13	0.981	0.975	0.985	0.939	0.987	0.981	0.983	0.972	0.988	0.969	0.972	0.951	1			
S14	0.964	0.947	0.991	0.967	0.975	0.983	0.964	0.974	0.993	0.993	0.985	0.961	0.972	1		
S15	0.994	0.994	0.972	0.915	0.988	0.967	0.981	0.943	0.97	0.954	0.953	0.919	0.98	0.949	1	
R	0.987	0.979	0.994	0.963	0.993	0.989	0.987	0.984	0.994	0.989	0.982	0.968	0.992	0.988	0.982	1

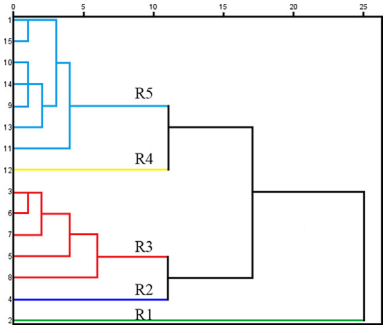

Fig. 2 HCA of 15 batches of raw VN samples.

is an unsupervised pattern recognition method based on a set of unclassified samples: the dendrogram was able to intuitively show the similarities and differences of the tested samples. As shown in Fig. 2, when the distance scale was approximately 11, fifteen samples were divided into five clusters, with considerable differences between them. The samples in group 5, which were formed with S1, S9, S10, S11, S13, S14 and S15, showed better similarity, which indicated that the samples in group 5 had the similar chemical constituents. All the samples from 15 batches VN, with their 22 common peaks, were assembled in a 15 × 22 data matrix for the PCA model. The R2 and Q2 values of the PCA score plot were 0.982 and 0.553, respectively, indicating that the established PCA model had a good fit and predictability. As shown in Fig. 3A, the different batches of VN samples do not exhibit a clear classification. As illustrated in Fig. 3B, each point in the load diagram represents a peak, and its distance from the origin represents the extent of its contribution to the overall distribution of the samples. The peaks No. 7, No. 8, No. 6, No. 4 and No. 11 were farther from the origin in the coordinate system, indicating that they had a greater contribution to the difference in quality among the VN samples.

The data of the samples were further analyzed using the supervised PLS-DA model to obtain the components of variability among the batches of samples (Campmajó et al., 2020; Zhao et al., 2020), the results showed that VN samples from different batches were divided into 5 groups, which confirmed the results of HCA analysis, as shown in Fig. 3C. The PLS-DA model of the 15 samples was generated to determine the potential markers from the 22 common peaks. The variable importance in projection (VIP), which represented the importance of each variable for distinguishing different regions, were shown in Fig. 3D. The components with VIP > 1 were usually considered as the potential classification markers (Windarsih et al., 2019). In total, six significant variables were obtained, which were peak 7 (veratramine), peak 8 (veratroylzygadenine), peak 6 (jervine), peak 11 (solanidine), peak 4 (veratrosine) and peak 10 (cucurbitacin B) in order of their projection importance values. These components were the main reasons for the differences among the 15 batches of samples and had a certain iconic effect, and the results were consistent with the results of the loadings plot in the PCA.

Based on the complexity of the chemical components of herbal medicine, fingerprinting techniques combined with chemical pattern recognition analysis can truly and graphically reflect the quality differences of herbal medicine and reveal the patterns among its complex components.

3.2. UPLC-MS/MS quantitative analysis of VN

Chromatographic fingerprinting of traditional Chinese medicine can reflect the wholeness and complexity of herbal medicine components, emphasizing the attribution of shared peaks and similarity evaluation, but the content of shared peaks of known components is difficult to determine, so the quantitative determination of multiple components using UPLC-MS/MS on the basis of fingerprinting can make up for the above deficiencies.

The linear calibration curves of peak areas (y) versus concentrations (x) were plotted for eight main active compounds. The regression coefficients (r^2) were > 0.9990 for the 8 compounds, indicating good linearity within a relatively wide range of concentrations. For precision, the RSD for the contents of

the 8 characteristic components ranged from 1.0 to 4.4 %. In the test of repeatability, the RSD values for all target analytes ranged from 1.7 to 4.6 %. The results indicated that the method is precise and repeatable. Stability, measured as RSD, was in the range 0.7–2.8 %, indicating that the samples were stable for 12 h. The recovery of each analyte ranged from 95.0 to 103.2 %. These results indicated that the method of sample preparation was acceptable in the current condition. The full scan monitoring chromatograms of the analytes are shown in Fig. 4. The validation data shown in Table 6 is considered to be satisfactory for the subsequent analysis of all samples.

The validated method was applied to the analysis of 15 batches of raw and processed VN samples. The contents of the 8 compounds in raw and processed VN samples are listed in Table 7. There were significantly differences in the contents of analytes between raw and processed samples. For example, compared with the raw VN, the total contents of veratrosine, jervine, resveratrol in most batches of processed VN is significantly increased. And the result of cyclopamine, veratramine, polydatin, apigenin is the opposite. As shown in Fig. 5, there are significantly decreased changes in contents of veratramine and polydatin. It can indicate two toxic components are sensitive to high temperature, and this phenomenon reveals that processing in high temperature may have a certain effect on reducing the toxicity of VN.

The UPLC-MS/MS results were further analyzed by PLS-DA. A three-dimensional (3 D) score plot of the PLS-DA was carried out to measure the differences in the content of raw and processed VN from different origins. There was no distinct zonal aggregation of VN from different origins, indicating that origin has no significant effect on the content of ingredients (Fig. 6). Raw and processed VN samples were clearly clustered in distinct region (Fig. 7). Constituents with

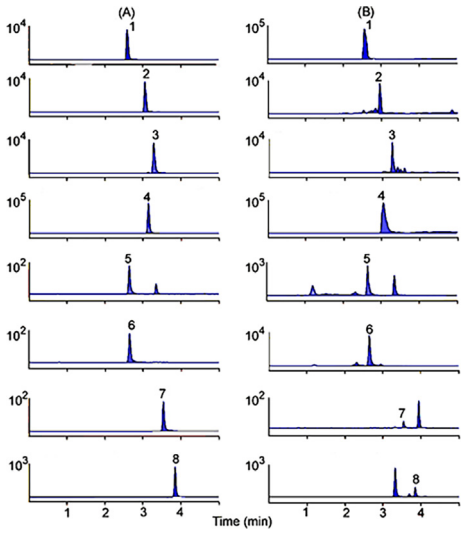

Fig. 4 Multiple reaction monitoring (MRM) chromatograms of Veratrosine (1), Jervine (2), Cyclopamine (3), Veratramine (4), Resveratrol (5), Polydatin (6), Quercetin (7), Apigenin (8). (A) standard solution, (B) VN sample.

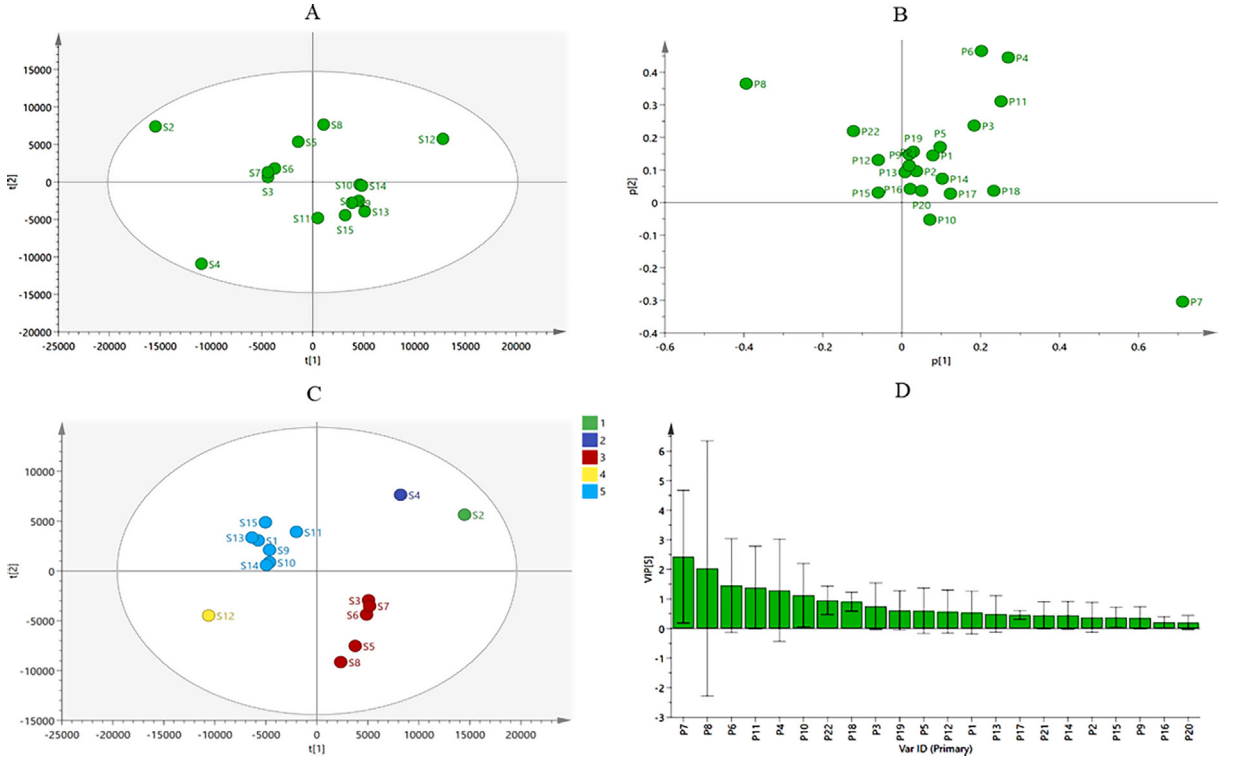

Fig. 3 Fngerprint analysis of chemometrics for 15 batches of VN. (A) The PCA score plot of 15 batches of VN. (B) The PCA loading plot. (C) The PLS-DA score plot. (D) The PLS-DA variable importance plot (VIP) histogram of 15 batches of VN. (2. Verdine, 3. pseudojervine, 4. Veratrosine, 5. Vibeissine, 6. Jervine, 7. Veratramine, 8. Veratroylzygadenine, 9. Cyclopamine, 10. Cucurbitacin B, 11. Solanidine).

Table 6 Regression equation, linear range, correlation coefficients (r^2), LLOQ, precision, repeatability, stability and recovery of 8 investigated analytes (n = 6).

Compounds	Regression equation	Linear range (ng/mL)	r2	LLOQ (ng/mL)	Precision RSD (%)	Repeatability RSD (%)	Stability RSD (%)	Recovery (%)
Veratrosine	y = 2138.1640x-3979.5370	2–400	0.9992	0.1	3.3	2.6	1.3	100.3
Jervine	y = 1424.9743x-2545.4059	2–400	0.9996	0.1	1.7	1.7	2.3	95.0
Cyclopamine	y = 2640.2316x-2230.1781	1–200	0.9991	0.1	1.0	2.8	1.7	96.3
Veratramine	y = 3634.1091x-45864.7029	15–3000	0.9990	0.1	1.1	2.8	1.9	96.0
Polydatin	y = 8.6428x-213.5339	35–7000	0.9993	0.2	4.0	2.6	2.4	103.2
Quercetin	y = 18.0939x-35.9843	2–400	0.9992	0.1	3.3	4.6	1.6	100.0
Apigenin	y = 21.6934x-37.2819	2–400	0.9991	0.2	4.4	2.3	2.8	102.9
Resveratrol	y = 50.2651x + 338.4728	8–1600	0.9995	0.1	1.3	1.8	0.7	99.4

Table 7 The contents of 8 compounds in *Veratrum nigrum* L. samples (μg/g).

Batch	Veratrosine		Jervine		Cyclopamine		Veratramine	
	Q	H	Q	H	Q	H	Q	H
1	4.36 ± 0.17	3.02 ± 0.18	2.80 ± 0.13	2.71 ± 0.10	1.12 ± 0.05	0.17 ± 0.01	121.08 ± 1.62	85.67 ± 3.03
2	2.99 ± 0.08	3.45 ± 0.07	2.42 ± 0.11	2.78 ± 0.08	1.24 ± 0.05	0.19 ± 0.01	126.88 ± 5.92	85.01 ± 1.72
3	2.91 ± 0.05	5.19 ± 0.16	3.10 ± 0.27	2.66 ± 0.11	1.79 ± 0.06	0.62 ± 0.28	90.83 ± 6.21	73.66 ± 4.62
4	2.88 ± 0.15	2.79 ± 0.23	3.54 ± 0.26	2.77 ± 0.12	1.32 ± 0.09	0.31 ± 0.01	103.15 ± 6.50	70.36 ± 3.27
5	6.65 ± 0.52	5.02 ± 0.21	2.41 ± 0.04	2.53 ± 0.11	1.43 ± 0.07	0.16 ± 0.01	99.22 ± 3.52	83.69 ± 3.06
6	9.07 ± 1.40	8.63 ± 2.77	2.25 ± 0.27	2.40 ± 0.10	2.39 ± 0.07	1.99 ± 0.02	96.19 ± 4.60	84.14 ± 4.09
7	4.26 ± 2.09	4.78 ± 0.22	2.91 ± 0.08	2.67 ± 0.03	1.67 ± 0.03	0.62 ± 0.56	97.59 ± 9.80	89.24 ± 1.49
8	7.02 ± 1.53	8.97 ± 2.80	2.63 ± 0.09	2.77 ± 0.14	2.55 ± 0.02	1.43 ± 0.69	96.52 ± 6.69	82.05 ± 2.10
9	3.46 ± 1.66	3.05 ± 1.31	2.87 ± 0.19	3.04 ± 0.10	1.49 ± 0.03	0.26 ± 0.02	96.40 ± 15.81	69.52 ± 7.10
10	3.28 ± 0.15	6.33 ± 0.36	3.23 ± 0.18	3.50 ± 0.04	1.74 ± 0.06	0.18 ± 0.02	81.16 ± 5.41	63.47 ± 1.85
11	3.71 ± 0.14	4.21 ± 0.18	3.09 ± 0.14	3.37 ± 0.18	1.95 ± 0.08	0.24 ± 0.03	81.79 ± 6.82	61.16 ± 1.18
12	8.64 ± 2.02	10.35 ± 0.43	2.28 ± 0.06	2.00 ± 0.09	2.53 ± 0.07	1.15 ± 0.77	98.20 ± 4.34	87.67 ± 1.00
13	2.92 ± 0.13	3.02 ± 0.09	2.73 ± 0.10	2.99 ± 0.14	1.51 ± 0.07	0.58 ± 0.55	104.16 ± 0.95	98.60 ± 2.96
14	2.72 ± 0.29	5.00 ± 0.17	3.63 ± 0.10	3.63 ± 0.06	1.72 ± 0.03	0.19 ± 0.02	84.89 ± 3.13	78.68 ± 5.79
15	2.80 ± 0.16	2.02 ± 0.07	3.17 ± 0.06	2.61 ± 0.07	1.33 ± 0.05	0.14 ± 0.02	120.49 ± 6.37	87.66 ± 6.40

(Remark: Q, raw samples; H, processed samples)

Batch	Polydatin		Quercetin		Apigenin		Resveratrol	
	Q	H	Q	H	Q	H	Q	H
1	93.44 ± 2.75	95.02 ± 5.85	0.07 ± 0.01	0.08 ± 0.01	0.83 ± 0.02	0.56 ± 0.01	5.69 ± 0.19	6.12 ± 0.13
2	83.59 ± 6.56	91.37 ± 4.05	0.09 ± 0.01	0.41 ± 0.49	0.62 ± 0.04	0.52 ± 0.01	5.54 ± 0.12	5.40 ± 0.09
3	105.98 ± 10.78	130.92 ± 2.15	0.24 ± 0.04	0.61 ± 0.06	0.76 ± 0.03	0.48 ± 0.02	2.58 ± 0.07	4.02 ± 0.16
4	115.45 ± 3.71	104.49 ± 8.99	0.26 ± 0.02	0.47 ± 0.01	1.28 ± 0.05	0.72 ± 0.03	5.31 ± 0.59	4.68 ± 0.16
5	141.64 ± 9.23	101.75 ± 3.30	0.09 ± 0.01	0.07 ± 0.01	0.93 ± 0.02	0.43 ± 0.01	3.49 ± 0.33	4.15 ± 0.20
6	128.75 ± 8.68	105.04 ± 4.66	0.50 ± 0.02	0.11 ± 0.01	0.65 ± 0.01	0.59 ± 0.01	3.98 ± 0.15	4.04 ± 0.10
7	93.84 ± 9.69	151.94 ± 15.16	0.16 ± 0.01	0.09 ± 0.01	0.86 ± 0.03	0.73 ± 0.01	5.75 ± 0.23	9.20 ± 0.46
8	92.35 ± 2.88	95.50 ± 8.25	0.25 ± 0.02	0.55 ± 0.03	0.81 ± 0.04	0.70 ± 0.03	3.31 ± 0.36	4.56 ± 0.10
9	81.20 ± 8.40	62.84 ± 4.18	0.18 ± 0.01	0.51 ± 0.02	0.67 ± 0.06	0.52 ± 0.01	2.62 ± 0.09	1.62 ± 0.02
10	130.90 ± 7.11	106.58 ± 4.30	0.15 ± 0.01	0.12 ± 0.02	0.96 ± 0.03	0.39 ± 0.01	3.33 ± 0.21	3.56 ± 0.28
11	119.37 ± 7.08	89.56 ± 6.63	0.14 ± 0.01	0.24 ± 0.01	0.86 ± 0.01	0.51 ± 0.02	2.34 ± 0.14	2.48 ± 0.08
12	126.41 ± 6.47	99.85 ± 5.44	0.18 ± 0.01	0.22 ± 0.02	0.70 ± 0.02	0.70 ± 0.03	4.17 ± 0.57	3.62 ± 0.30
13	137.04 ± 8.75	89.88 ± 4.82	0.11 ± 0.01	0.09 ± 0.02	1.06 ± 0.01	0.70 ± 0.01	7.76 ± 0.27	4.95 ± 0.07
14	113.61 ± 7.99	102.42 ± 2.16	0.21 ± 0.01	0.14 ± 0.01	0.99 ± 0.03	0.62 ± 0.02	2.75 ± 0.12	2.78 ± 0.02
15	90.99 ± 5.18	68.98 ± 2.90	0.07 ± 0.01	0.09 ± 0.02	0.80 ± 0.10	0.43 ± 0.01	5.65 ± 0.25	4.32 ± 0.17

(Remark: Q, raw samples; H, processed samples)

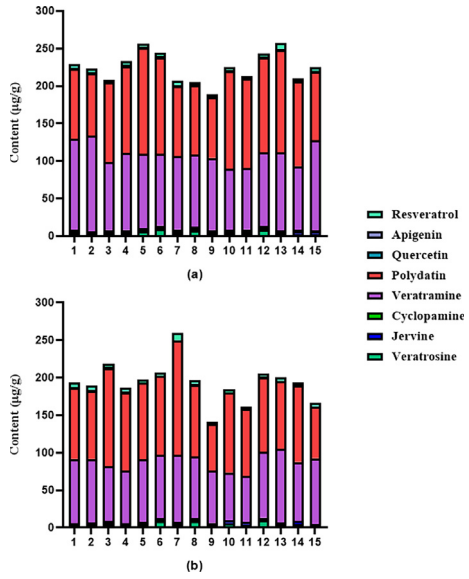

Fig. 5 Contents of 8 components in different batches of raw and processed VN (μg/g): (A) raw; (B) processed.

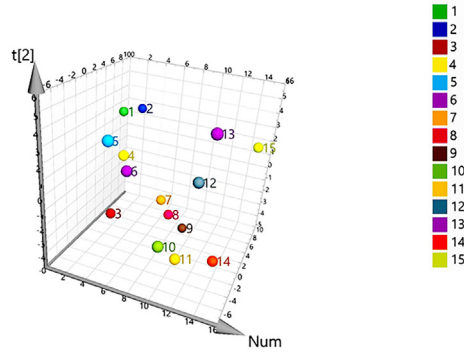

Fig. 6 Partial least squares discriminant analysis (PLS-DA) 3D score scatter plot for 15batches of raw VN.

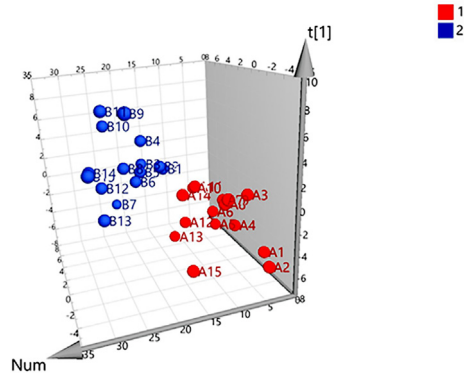

Fig. 7 Partial least squares discriminant analysis (PLS-DA) 3D score scatter plot for raw (1) and processed VN. (2).

large loading values can be considered as markers, which contributed clearly to the classification of the samples. In the present study, potential bioactive ingredients such as veratramine and polydatin with VIP > 1 were identified as compounds that could be used to differentiate between the raw and processed VN samples, as illustrated in Fig. 8. The results showed that the chemical composition of VN samples was altered after processing. The above components contributed greatly to sample classification and may be the material basis for the change of clinical efficacy associated with herb processing.

4. Conclusions

In this study, a new method of fingerprint analysis combined with qualitative analysis was established to evaluate the quality of VN, the results of fingerprint showed that the 15 batches of samples contained 22 common peaks and their similarity results were > 0.963. The chromatographic data of multiple fingerprints of VN were balanced and analyzed by chemometric methods including HCA, PCA and PLS-DA. The results of the three analyses were validated against each other, which systematically explained the intrinsic quality characteristics of VN. Further analysis of PLS-DA indicated that potential chemical markers for the classification of VN from different origins were veratramine, veratroylzygadenine, jervine, solanidine, veratrosine and cucurbitacin B. In addition, 8 bioactive compounds in VN samples were quantified by UPLC-MS/MS, of which veratramine and polydatin were identified, based on PLS-DA, as key compounds that can be helpful to distinguish between raw and processed VN samples. The methods, combining UPLC-Q-TOF/MS, fingerprint and UPLC-MS/MS, are established for the identification of raw VN and its processed products, which provided the basis for the quality evaluation of VN.

CRediT authorship contribution statement

Guangzhe Yao: Methodology, Data curation, Software, Writing – original draft. **Mengxuan Wu:** Methodology, Data curation, Software. **Minglei Ge:** Methodology, Data curation, Software. **Mengmeng Zhang:** Methodology, Data curation, Software. **Xiunan Cao:** Methodology, Data curation, Software. **Yameng Zhu:** Methodology, Data curation, Software. **Shujie Wei:** Methodology, Data curation, Software. **Yanxu Chang:** Writing – review & editing. **Huizi Ouyang:** Writing – review & editing. **Jun He:** Conceptualization, Funding acquisition, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

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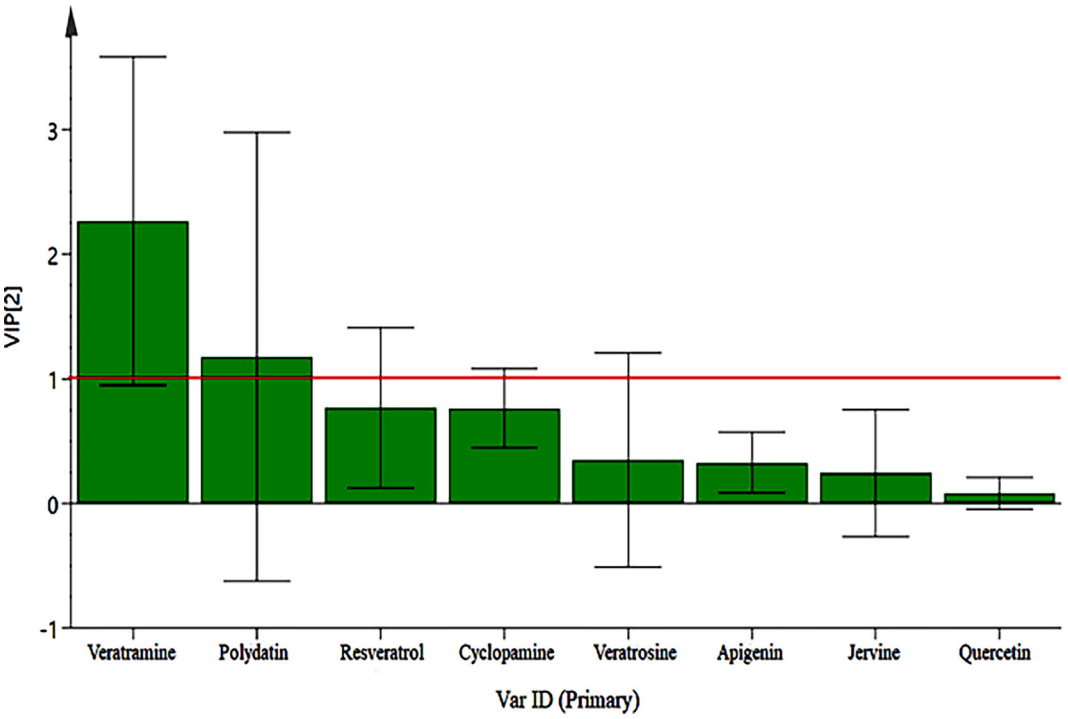

Fig. 8 VIP plot of PLS-DA about raw and processed VN samples (1. Veratramine, 2. Polydatin, 3. Resveratrol, 4. Cyclopamine, 5. Veratrosine, 6. Apigenin, 7. Jervine, 8. Quercetin.).

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