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Alkaloid profiling of crude and processed *Veratrum nigrum* L. through simultaneous determination of ten steroidal alkaloids by HPLC–ELSD

Yue Cong[a,b], Yu-Bo Zhou[a], Jing Chen[a], Yi-Mei Zeng[a], Jin-Hui Wang[a,c,*]

[a] *School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, PR China*
[b] *Institute of Traditional Chinese Medica, School of Pharmacy, Henan University, Kaifeng, PR China*
[c] *Shihezi University, Shihezi, PR China*

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ABSTRACT

Veratrum nigrum L., a traditional Chinese herb, has been used for treatment of hypertension, blood-stroke, excessive phlegm, epilepsy, etc. Steroidal alkaloids were well-known as both bioactive and toxic constituents of *Veratrum* species, the toxicity of which the traditional processing procedure can reduce. To reveal the mechanism of processing *V. nigrum* L., a high performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) method was developed for the simultaneous determination of ten steroidal alkaloids in crude and processed *V. nigrum* L., comparison with ultrasound extract of crude *V. nigrum* L. With a Venusil XBP-C_{18} analytical column, the analytes were separated efficiently using the mobile phase consisted of (A) 0.03% aqueous triethylamine (TEA) and (B) acetonitrile in a gradient program. The parameters for ELSD were set: S.C. (Spray Chamber) = 35 °C, D.T. (Drift Tube) = 70 °C, GAS = 50 psi. All calibration curves showed good linear regression ($\gamma \geq 0.9990$) within the tested range. Additionally, reproducibility for the quantification of ten alkaloids in *V. nigrum* L. with intra- and inter-day variations of less than 5.0% was observed. The obtained alkaloid profiles performed by this newly established method, provided valuable information for the differentiation of crude and processed *V. nigrum* L. and for the explanation of the different toxicity.

1. Introduction

Traditional Chinese medicines are gaining increasing popularity worldwide for disease treatment in recent years. While quite a mount of the effective herbs also demonstrated toxic side effects, such as *Veratrum nigrum* L., *Aconitum carmichaeli* Debx, *Evodia rutaecarpa* (Juss.) Benth and so on. The traditional processing procedures, under the guidance of the theory of traditional Chinese medicine (TCM) science, have been proved to be important to reduce the toxicity or enhance the curative effect before the herbs can be used in the prescription of traditional medicines. Thousands of years history on herbs practice have proved that toxic side effects of herbs could be reduced and even eliminated after being properly processed with parching, steaming or soaking [1,2].

V. nigrum L., a traditional Chinese herb, has been used for treatment of hypertension, blood-stroke, excessive phlegm, epilepsy, etc. However, it is well-known for *V. nigrum* L. as a poisonous traditional Chinese medicine, and water extract of it has toxic and irritant activity on the digestive tract mucosa, nucleus nervi vagi, and central nervous system [3–5]. Thus, it is essential for the experiment study of the decreasing toxicity on the processing procedures of *V. nigrum* L. by analysis of components of processed and crude *V. nigrum* L., in combination with acute toxicity test [6].

This study was conducted to explore the differences for the dissolution ratios of chemical constituents in the roots and rhizomes of *V. nigrum* L. and its processed products, by rice vinegar or mixture of rice flour and water [7–11]. It has demonstrated that the major bioactive and toxic ingredients present in this TCM herb are steroidal alkaloids by chemical and pharmacological studies [1,12]. Steroidal alkaloids in *V. nigrum* L. were proved to possess significant antihypertensive and antifungal effects, while at the same time cause skeletal muscle toxicity, neuro toxicity, and mutagenic potential [13–15], so it is reasonable and logical to be considered as useful markers relevant to biological activities and toxicities for the quality control and toxicological study of the roots and rhizomes of *V. nigrum* L. Moreover, both quality and quantity controls of the major active alkaloids in this herb have always been an important issue to ensure its effective and safe clinical usefulness.

* Corresponding author at: School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, PR China. Tel.: +86 24 23986478.
E-mail address: Wjh.1972@yahoo.com.cn (J.-H. Wang).

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However, the absence of a chromophore in these steroidal alkaloids hamper their detection with a UV detector. On the other hand, evaporative light scattering detector (ELSD) has been successfully applied for steroidal alkaloids profiling and quantification [16]. Thus, a HPLC method coupled with ELSD was employed in this study.

In the present report, a HPLC–ELSD method has been developed for simultaneous determination of ten steroidal alkaloids, namely verdine(**1**), pseudojervine(**2**), veratrosine(**3**), 1β, 3α-dihydroxy-5β-jervanin-12-en-11-one(**4**), jervine(**5**), veratramine(**6**), 12β-hydroxyl veratroylzygadenine(**7**), veratroylzygadenine(**8**), veramarine(**9**), and rubijervine (**10**) in the Chinese herbal medicine *V. nigrum* L. and its different processed samples for accessing the mechanism of processing drugs (Fig. 1).

2. Experimental

2.1. Chemicals and materials

Acetonitrile (HPLC-grade) was purchased from Tianjing Kangkede Technology Co. Ltd. (Tianjing, China) and deionized water was from Hangzhou Wahaha Co. Ltd. (Hangzhou, China). Triethylamine (TEA) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Other solvents from Tianjing Kangkede Technology Co. Ltd. (Tianjing, China) were of analytical grade.

The plant material of *V. nigrum* L. was purchased from a herbal market in Shenyang city, Liaoning province, China and was identified by Professor Qishi Sun (Shenyang Pharmaceutical University). A voucher specimen (No. 20040710) is deposited in School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University. The reference standards of 1–10 steroidal alkaloids were isolated previously from the crude alkaloids of *V. nigrum* L. by author [17,18], structures of which were established based on spectroscopic analyses compared with the reference data [19–24]. The purities of these steroidal alkaloids were determined to be above 97% by normalization of the peak areas detected by HPLC–ELSD and confirmed by EI-MS, ESI-MS, and NMR spectroscopy.

2.2. Apparatus and chromatographic conditions

Analyses were performed using an Laballiance series III HPLC system (Thermo Electron Corporation, USA) equipped with model 200es ELSD system (SOFTA Corporation, USA) coupled with an WYK-16b4 air compressor (Tianjing Dayin Apparatus Factory, Tianjing, China). The chromatography was carried out on a Venusil XBP-C_{18} column (5 μm, 200 mm × 4.6 mm) at a column temperature of 30 °C and flow rate of 1 ml/min using (A) acetonitrile and (B) water containing 0.03% triethylamine (TEA) as mobile phase with a linear gradient (see Table 1). Parameters for the ELSD were set: S.C. (Spray Chamber) = 35 °C, D.T. (Drift Tube) = 70 °C, GAS = 50 psi.

2.3. Sample preparation

Three different preparation processes were tested: (A) aqueous extract of crude *V. nigrum* L., (B) aqueous extract of *V. nigrum* L. processed with rice vinegar, (C) aqueous extract of *V. nigrum* L. processed with mixture of rice flour and water. The concrete procedure of each was described as following:

(A) The crude roots and rhizomes of *V. nigrum* L. (100 g) were milled into powder and dried at 80 °C for 4 h before determination of the steroidal alkaloids.

(B) The dried roots and rhizomes of *V. nigrum* L. (100 g) cut to pieces of 15 mm were mixed with a blend of rice vinegar (18 g) and water (36 g) for one night. When the rice vinegar was absorbed completely, the mixture was roasted until dry [3,7,8,25], finally air dried and milled into powder. The obtained powder was further dried at 80 °C for 4 h before determination.

(C) The dried roots and rhizomes of *V. nigrum* L. (100 g) cut to pieces of 15 mm were blended with mixture of rice flour (8 g) and water (400 g) to soak for 24 h, then washed quickly by water to roast until dry [3,7–9,26], finally air drying and milled into powder. The obtained powder was further dried at 80 °C for 4 h before determination.

Approximately 2.0 g of dried herb (A)–(C) were respectively accurately weighed and separately added to a round-bottom flask containing 20 ml water. The mixtures were boiled on an electric heater under reflux for 2 h. After evaporating water to dryness by a rotary evaporator, residue was dissolved in the mixed liquor of MeOH–$CHCl_3$ (1:1) in a 5 ml volumetric flask, and then filtrated through a 0.45 μm millipore filter to get the sample (A)–(C).

Approximately 2.0 g of crude dried herb was accurately weighed and added to a round-bottom flask containing 2 ml 14% ammonia water to infiltrate for 2 h, then 20 ml the mixed liquor of MeOH and $CHCl_3$ (1:1) was added in this round-bottom flask to ultrasonicated for 20 min. After evaporating to dryness by a rotary evaporator, residue was dissolved in the mixed liquor of MeOH and $CHCl_3$ (1:1) in a 5 ml volumetric flask, and then filtrated through a 0.45 μm millipore filter to get the sample (D).

3. Validation of the method

3.1. Calibration curves

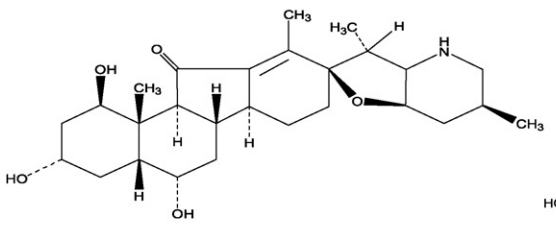
A stock solution containing ten analytes was prepared by dissolving the reference compounds in the mixed liquor of MeOH–$CHCl_3$ (1:1) and then was diluted with the mixed liquor of MeOH–$CHCl_3$ (1:1) to appropriate concentrations for establishing calibration curves. Solutions containing different concentrations of the ten analytes were injected in triplicate. Calibration curves were plotted logarithm using peak area versus concentration for each analyte.

3.2. Limits of detection and quantitation

Stock solution containing ten reference compounds was diluted to a series of appropriate concentrations with the mixed liquor of MeOH and $CHCl_3$ (1:1), and an aliquot of the diluted solutions was injected into HPLC for analysis. The limit of detection (LOD) and limit of quantification (LOQ) for each analyte was calculated with corresponding standard solution on the basis of a signal-to-noise ratio (S/N) of 3 and 10, respectively.

3.3. Precision and stability

Intra- and inter-day variations were determined for precision of the developed method. Certain concentrations of standard and sample were tested. For intra-day variability test, the standard solution was analyzed for six times within 1 day, and the inter-day reproducibility was determined with six individual sample solutions for 3 consecutive days. Stability study was performed with sample solution on 2 consecutive days (n = 8). Variations were expressed by relative standard deviations (R.S.D.).



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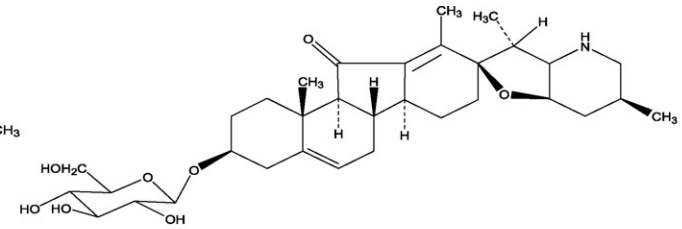

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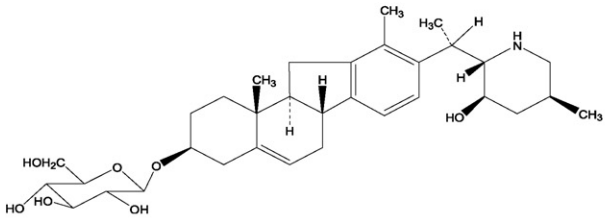

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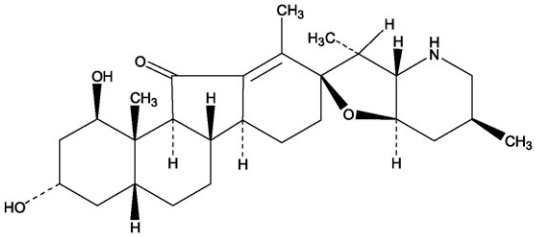

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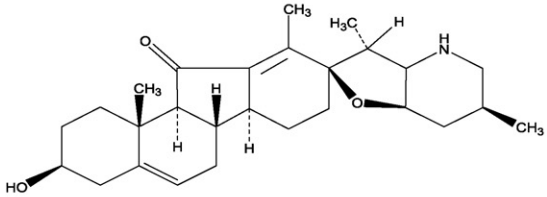

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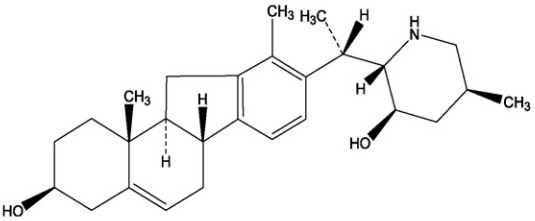

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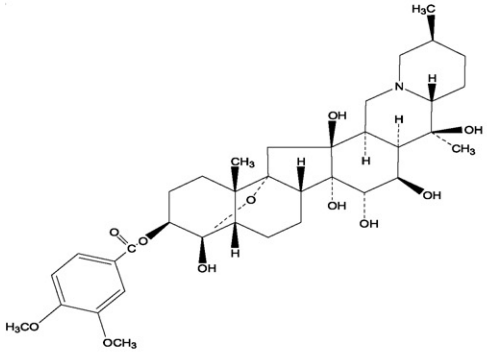

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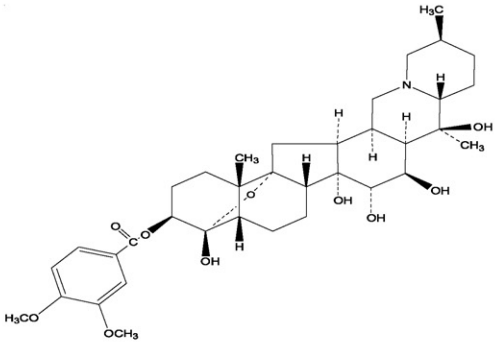

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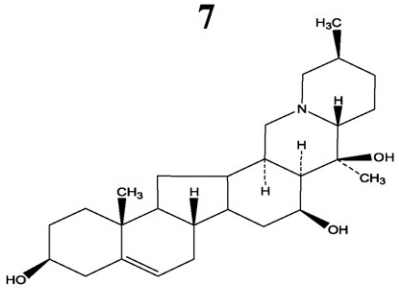

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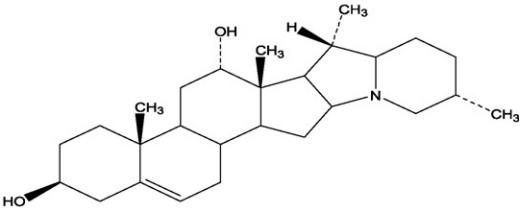

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Fig. 1. Structures of compounds 1–10.

Table 1
Time program of the gradient elution

Time (min)	Flow	CH_3CN	0.03% $N(CH_2CH_3)_3$
0	1	30	70
15	1	36	64
23	1	36	64
33	1	40	60
41	1	50	50
51	1	60	40
61	1	70	30
71	1	100	0
78	1	100	0

4. Results and discussion

4.1. Optimization of separation conditions

In the present study, ten major steroidal alkaloids were chosen as chemical markers to simultaneously evaluate the quality of crude and processed *V. nigrum* L. As shown in Fig. 1, the compounds have very broad range of polarity, so gradient elution was carried out to separate these components in *V. nigrum* L. Several mobile phases including methanol–water and acetonitrile–water in combination with acetic acid were examined. Finally, it was found that 0.03% (v/v) aqueous TEA–acetonitrile system gave the best separation of ten steroidal alkaloids, and all ten compounds could be eluted with baseline separation in 78 min. Representative chromatograms for the ten standard analytes and for herb samples were shown in Fig. 2. Fig. 2(E) displayed that the ten standard analytes were well separated and the resolution between any two compounds was greater than 1.0. Other compounds in the sample do not interfere with the analysis of the ten steroidal alkaloids, as shown in Fig. 2(A)–(D). The chromatographic peaks were identified by comparing their retention time with that of each reference compound, which was eluted in parallel with a series of mobile phases. In addition, spiking samples with the reference compounds showed no additional peaks, which further confirmed the identities of the analytes' peaks (Fig. 3).

4.2. Optimization of ELSD parameters

The quantitation of investigated steroidal alkaloids was achieved by using an 200es ELSD (SOFTA, USA). The parameters of ELSD including spray chamber temperature, drift tube temperature, and nebulizing gas pressure were optimized to obtain the best signals when ratio of signal to noise (S/N) was taken as a measurement according to the data computed with the ELSD software. Compound **6** was selected as a model alkaloid for optimizing ELSD conditions because it was contained in most samples. Spray chamber temperature, drift tube temperature, and nebulizing gas pressure for

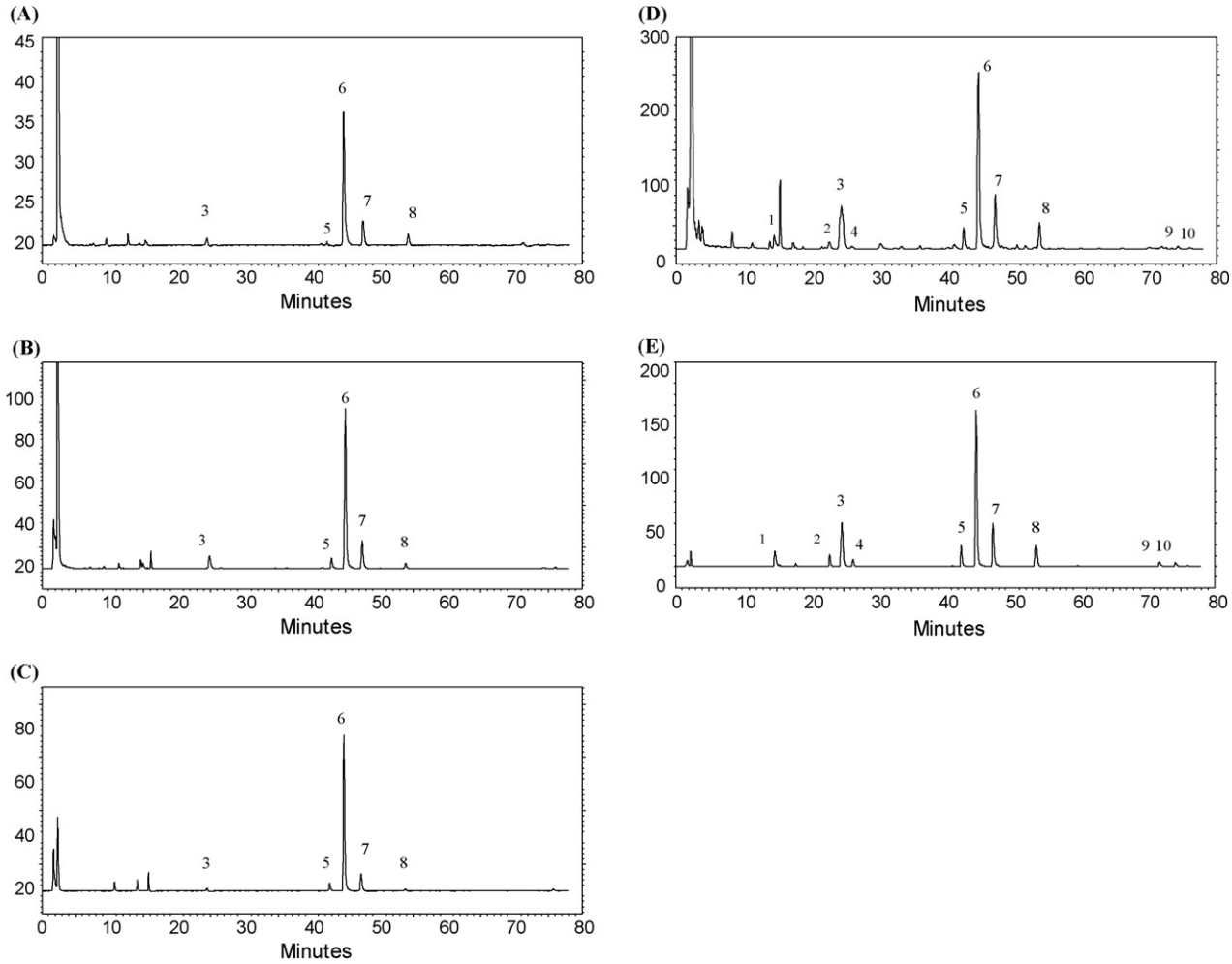

Fig. 2. Typical HPLC chromatograms of (A) water extract of crude *Veratrum nigrum* L.; (B) water extract of *V. nigrum* L. processed with rice vinegar; (C) water extract of *V. nigrum* L. processed with mixture of rice flour and water; (D) ultrasonic extract of crude *V. nigrum* L.; (E) mixed standards; Column: Venusil XBP-C_{18} column (200 mm × 4.6 mm, 5.0 μm), temperature of 30 °C; detector: ELSD, S.C. = 35 °C, D.T. = 70 °C, GAS = 50 psi.

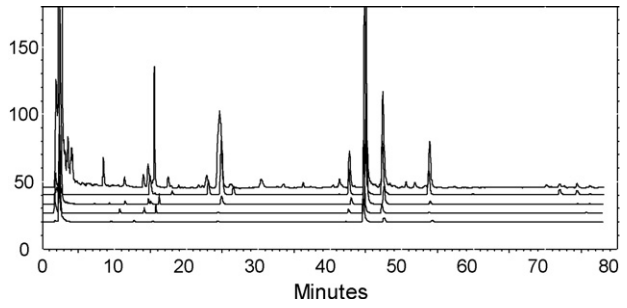

Fig. 3. Stack view of HPLC chromatograms of (D) ultrasonic extract of crude *V. nigrum* L.; (E) mixed standards; (B) water extract of *V. nigrum* L. processed with rice vinegar; (C) water extract of *V. nigrum* L. processed with mixture of rice flour and water; (A) water extract of crude *V. nigrum* L.; (from up to down).

the detector was evaluated systematically from 30 to 45 °C, from 60 to 80 °C, and from 40 to 60 psi, respectively. The effect of spray chamber temperature and nebulizing gas pressure of ELSD based on S/N of **6** showed that the optimal parameters were identified as 35 °C and 50 psi. In general, the highest signal should be obtained at drift tube temperature 60 °C, but in the present study the baseline was unstable and noise was high. Therefore, the optimal drift tube temperature was 70 °C according to the effect of drift tube temperature of ELSD on S/N of the analyte. Accordingly, the optimized parameters of ELSD were 35 °C for spray chamber temperature, 70 °C for drift tube temperature, and 50 psi for nebulizing gas pressure.

4.3. Validation of the method

It has been generally observed that the detector response, as measured by peak area, varies exponentially with the mass of analyte [27], and this behavior can be mathematically expressed in logarithmic form. Present experimental results showed that the logarithm of peak area of each standard was linearly correlated to the logarithm of injected concentration within a particular range (Table 2). As shown in Table 2, all calibration curves showed good linear regression ($\gamma \geq 0.999$) and the LOD was less than 0.28 μg, indicating that this HPLC–ELSD method is precise and sensitive for the quantitative evaluation of major active steroidal alkaloids in *V. nigrum* L. Validation studies of the method proved that this assay had good reproducibility, and the overall intra-day and inter-day variations were less than 5.0% for all analytes (R.S.D., 1.35–4.13%). It was also found that the analytes in the sample solution stable in 2 days with a relative standard deviation of 1.52–3.58%.

4.4. Quantitative determination of V. nigrum L.

Steroidal alkaloids in *V. nigrum* L. possess significant antihypertensive and antifungal effects, at the same time which produce skeletal muscle toxicity, neuro toxicity and mutagenic potential. Therefore, these ten steroidal alkaloids were adopted as chemical markers to establish a method for the quality control and toxicological study of *V. nigrum* L. The current method was utilized to analyze the ten steroidal alkaloids in four samples in triplicate to compare the different concentrations of the ten steroidal alkaloids for discussing processing mechanism on *V. nigrum* L. (Table 3). It was found that there were remarkable differences between preprocessing and processed, in terms of concentrations of the ten steroidal alkaloids. Among the ten steroidal alkaloids, compounds **3**, **5**–**8** were found to be contained in the samples (A)–(C). In terms of the chromatographic results that concentrations of compounds **3**, **5**–**8** in the sample (B) were higher than those in the samples (A) and (C), it was demonstrated that dissolution rates of steroidal alkaloids of *V. nigrum* L. processed by rice vinegar increased in water. Thus, the processing *V. nigrum* L. by rice vinegar was potentiated without de-toxic effect, proven by acute toxicity test [6], which indicated that the processing procedure with rice vinegar is not suitable to raw material of *V. nigrum* L. In addition, contents of compounds **3** and **8** in the sample (A) were higher than that in the sample(C), and concentrations of compounds **5**–**7** in the sample (A) were lower than that in the sample (C), in combination with the result of acute toxicity test [6] that toxicity of the sample (C) was less than that of the sample (A), by which can be concluded that the processing *V. nigrum* L. by the mix of rice flour and water can reduce the toxicity in some extent. The reason still need further investigation.

Table 2
Linear regression and precision data of the ten analytes in *V. nigrum* L.

Analytes	Linear regression				Precision	
	Calibration curves	γ	Linear range (μg)	LOD (μg)	Intra-day R.S.D.(%)	Inter-day R.S.D.(%)
1	$Y = 1.30x + 6.37$	0.9991	2.00–10.00	0.19	1.35	2.20
2	$Y = 1.46x + 6.23$	0.9997	2.00–10.00	0.21	1.41	2.05
3	$Y = 0.74x + 6.10$	0.9992	8.00–40.00	0.28	2.11	3.26
4	$Y = 1.42x + 6.83$	0.9990	0.60–3.00	0.17	2.41	4.07
5	$Y = 1.40x + 6.31$	0.9993	2.00–10.00	0.22	1.43	2.45
6	$Y = 0.94x + 6.41$	0.9998	8.00–40.00	0.14	1.78	2.72.
7	$Y = 1.32x + 6.46$	0.9990	3.00–15.00	0.20	2.03	3.15
8	$Y = 1.33x + 6.39$	0.9995	2.00–10.00	0.18	2.79	4.13
9	$Y = 1.63x + 6.40$	0.9990	1.00–5.00	0.19	1.94	3.05
10	$Y = 1.05x + 5.89$	0.9991	1.00–5.00	0.16	1.55	3.66

In the calibration curves, $Y = \lg A$, $x = \lg C$ (A: peak area, C: mg/ml; lg: logarithm) and γ is the correlation coefficient. LOD refers to limit of detection. R.S.D. refers to relative standard deviation.

Table 3
Contents of ten analytes in samples of *V. nigrum* L (mg/g)

Sample	1	2	3	4	5	6	7	8	9	10
A	nd[a]	nd	0.012	nd	0.048	0.252	0.128	0.078	nd	nd
B	nd	nd	0.121	nd	0.233	1.050	0.349	0.118	nd	nd
C	nd	nd	0.005	nd	0.148	0.751	0.198	0.043	nd	nd
D	0.649	0.712	5.941	0.165	0.825	4.521	1.382	0.910	0.233	0.195

[a] Not detected.

5. Conclusions

A new analytical method for qualification and quantification of steroidal alkaloids in *V. nigrum* L. was evaluated to be precise and accurate. Raw materials and processed materials of *V. nigrum* L. were assayed with this method. The direct analytical method is particularly applicable for toxicity comparison between the crude and processed *V. nigrum* L. by the detection of steroidal alkaloids, which also provides a new tool for the assessment of quality of *V. nigrum* L.

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