

**Chemical Fingerprint and Quantitative Analysis for the Quality Evaluation of  
*Vitex negundo* Seeds by RP-HPLC coupled with Hierarchical Clustering Analysis**

Zhiheng Shu<sup>1,2,†</sup>, Xiuqing Li<sup>2,†</sup>, Khalid Rahman<sup>3</sup>, Chengjian Zheng<sup>2\*</sup> and Luping Qin<sup>2\*</sup>

<sup>1</sup>School of Pharmacy, Ningxia Medical University, Yinchuan 750004, P. R. China

<sup>2</sup>Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai 200433, P. R. China

<sup>3</sup>Faculty of Science, School of Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, England, UK

†Zhiheng Shu and Xiuqing Li contributed equally to this manuscript; both should be considered as first author.

**Running Title:** Quality evaluation of *Vitex negundo* seeds by HPLC

**Correspondence:**

Dr. Cheng-Jian Zheng

Mailing address: No.325 Guohe Road, Yangpu District, Shanghai 200433, China

Fax: +86-21-81871305

E-mail: zhengchengjian@smmu.edu.cn

Prof. Lu-Ping Qin

Mailing address: No.325 Guohe Road, Yangpu District, Shanghai 200433, China

Fax: +86-21-81871309

E-mail: qinsmmu@126.com

**Abbreviations:** **DAD**, diode array detection; **HCA**, hierarchical cluster analysis;

**TCM**, traditional Chinese medicine; **PA**, peak area; **RPA**, relative peak area; **t<sub>R</sub>**,

retention time; **RRT**, relative retention time; **VNL**,

6-hydroxy-4 $\beta$ -(4-hydroxy-3-methoxyphenyl)-3 $\alpha$ -hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde; **VN-2**, vitedoin A; **VN-5**, vitedoamine A; **VN-12**, vitexdoin A

**Key words:** *Vitex negundo* seeds, Chromatographic fingerprint, Liquid chromatography, Hierarchical clustering analysis, Quality evaluation

## Abstract

A simple and efficient method was developed for the chemical fingerprint analysis and simultaneous determination of four phenylnaphthalene-type lignans in *Vitex negundo* seeds using high-performance liquid chromatography with diode array detection. For fingerprint analysis, 13 *V. negundo* seed samples were collected from different regions in China, and the fingerprint chromatograms were matched by the computer-aided *Similarity Evaluation System for Chromatographic Fingerprint of TCM* (Version 2004A). A total of 21 common peaks found in all the chromatograms were used for evaluating the similarity between these samples. Additionally, simultaneous quantification of four major bioactive ingredients was conducted to assess the quality of *V. negundo* seeds. Our results indicated that the contents of four lignans in *V. negundo* seeds varied remarkably in herbal samples collected from different regions. Moreover, the hierarchical clustering analysis grouped these 13 samples into three categories, which was consistent with the chemotypes of those chromatograms. The method developed in this study provides a substantial foundation for the establishment of reasonable quality control standards for *V. negundo* seeds.

## **1 Introduction**

Traditional Chinese medicine (TCM) is an empirical drug system under the guidance of classical TCM theory. Currently, TCM has increasingly attracted worldwide attention as an alternative therapeutic modality due to its low toxicity, general side effects, convinced efficacy and rare drug tolerance [1]. However, the quality of medicinal plants may be considerably affected by many factors, such as the plant origins, harvesting time, and processing method, which ultimately result in their chemical complexity and variation. Therefore, quality evaluation is a key issue in the process of TCM modernization and globalization [2]. While one or two marker compounds are generally quantified to evaluate the quality and potency of TCM, they cannot always reflect the overall quality of TCM or reveal the comprehensive chemical profile of those herbal drugs. Therapeutic effects of TCM are usually constituted as a result of multiple bioactive components [3]. Therefore, efficient and characteristic quality assessment methods are urgently required for TCM. As a holistic approach for quality control, chemical fingerprint analysis, which emphasizes the entire profile of chemical components and their relative concentrations, has been internationally accepted by the World Health Organization (WHO), the Food and Drug Administration of the U.S. (FDA), the Chinese State Food and Drug Administration (SFDA) and other authorities [4]. Among all the techniques used for fingerprint analysis, high-performance liquid chromatography (HPLC) is the principal method commonly used for establishing fingerprints due to its simplicity, wide stability and durability [5, 6]. Because fingerprint analysis is regarded as an effective

method for evaluating batch-to-batch consistency and ensuring quality control of TCM, the combination of fingerprint and quantitative analyses of active components would be quite powerful in revealing the real quality of TCM [7, 8].

*Vitex negundo* L. is an aromatic shrub distributed widely in China, Japan, Indonesia, East Africa and South America [9]. The seeds of *V. negundo*, named “Huang-Jing-Zi” in China, have been used for the treatment of analgesia, rheumatism, arthritis and chronic bronchitis in folk medicine [10, 11]. Phytochemical and pharmacological studies on *V. negundo* seeds revealed the presence of bioactive flavonoids [12], lignans [9, 13] and terpenoids [13, 14]. Among these compounds, the phenylnaphthalene-type lignans have been particularly revealed to possess versatile bioactivities, including anti-inflammatory, anti-nociceptive, anti-oxidant, anti-osteoporotic, and anti-tumor activities [15-19]. Our phytochemical investigations on this plant afforded a series of phenylnaphthalene-type lignans, including 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde, vitedoin A, vitedoamine A, as well as 10 new compounds, including vitexdoin A-I and vitedoamine B [9, 20]. Our further pharmacological studies have also demonstrated that phenylnaphthalene-type lignans were the principal bioactive components [9, 11, 20, 21]. In particular, 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde (**VNL**), vitedoin A (**VN-2**), vitedoamine A (**VN-5**) and vitexdoin A (**VN-12**), four major phenylnaphthalene-type lignans in *V. negundo* seeds (**Fig. 1**), have been reported to possess promising bioactivities, including anti-inflammatory,

analgesic, anti-tumor, and anti-oxidative properties [13, 16, 17, 22-24].

Because phenylnaphthalene-type lignans possess convincing pharmacological activities, it may be concluded that that phenylnaphthalene-type lignans serve as the major fraction responsible for the bioactivities of *V. negundo* seeds. Therefore, the rapid and direct characterization of phenylnaphthalene-type lignans in crude plant extracts is crucial for ensuring the efficacy and quality of *V. negundo* seeds. Unfortunately, no report has provided such a characterization to date.

In this pioneering study, a HPLC-DAD method was performed to establish the chemical fingerprints and simultaneously provide a determination of the above-mentioned four major phenylnaphthalene-type lignans of *V. negundo* seeds. However, the complex and multivariate data sets of fingerprint chromatograms often led to minor differences between similar chromatograms with comprehensive chemical complexities being overlooked [25]. Therefore, hierarchical clustering analysis (HCA), a chemical pattern recognition method, was employed to define the classification and evaluate the interspecific differentiation of *V. negundo* seed samples. We hope that our established method will be helpful for the future quality control of *V. negundo* seeds.

## **2 Materials and methods**

### **2.1 Materials and chemicals**

A total of 13 *Vitex negundo* seed samples were collected from the provinces of Sichuan, Shanghai, Jiangxi, Zhejiang, Jiangsu, Fujian, Hunan, Yunnan and Shandong in China (**Table 1**). All samples were identified and confirmed by Professor Luping

Qin (Department of Pharmacognosy, School of Pharmacy, Second Military Medical University). Voucher samples were stored at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University in Shanghai, China. All samples were dried at 22-25°C, pulverized and sieved through a 24-mesh (0.850 mm) stainless-steel sieve before extraction. Sample No. S13 (collected from the Sichuan province) was used for the method optimization studies.

The 95% ethanol (analytical grade) and formic acid (analytical grade) were obtained from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Fisher Scientific International Inc. (Fair Lawn, New Jersey, USA). High purity water was purchased from the Hangzhou Wahaha Group Co., Ltd. (Hangzhou, China).

Four chemical standards, namely, 6-hydroxy-4 $\beta$ -(4-hydroxy-3-methoxyphenyl)-3 $\alpha$ -hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde (VNL), vitedoin A (VN-2), vitedoamine A (VN-5), and vitexdoin A (VN-12), were previously isolated from the ethanol extract of *V. negundo* seeds in our laboratory. The purities of these standards were higher than 98%, as analyzed by the HPLC area normalization method. Their structures were confirmed by IR, ESI-MS, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR [9, 11], as shown in **Fig. 1**.

## **2.2 Apparatus and chromatographic condition**

The Agilent 1200 HPLC system used in our study consists of a vacuum degasser, binary pump, autosampler, thermostated column compartment, and diode array detector (DAD) (Agilent, USA). System control and data analysis were processed

with the Chemstation Software program (version A.10.02). The chromatographic separation was performed on an Agilent Zorbax Extend-C18 column (250 mm × 4.6 mm, 5 μm), using acetonitrile (A) and a 0.1% aqueous formic acid solution (v/v, B) as the mobile phase at a flow rate of 0.8 mL/min. The gradient program was set as follows: 0–14 min, 8–15% A; 14–35 min, 15–25% A; and 35–70 min, 25–55% A. The chromatogram was monitored at a wavelength of 254 nm during the experiment. The column temperature was maintained at 30°C, and the injection volume of each sample and standard solution was set at 20 μL. All solutions were filtered through a 0.45 μm membrane filter before HPLC analysis.

### **2.3. Sample and standard solution preparation**

A total of 1.0 g of dried powder was put into a 50 mL Erlenmeyer flask. Approximately 30 mL of an 80% v/v methanol solution was added to the flask. Following ultrasonic extraction at 60°C for 90 min, the extracted solution was concentrated under vacuum at 50°C, and the dried extract was dissolved in 30 mL of 50% v/v methanol, followed by sonication for 10 mins. After adding solvent to the original volume, the sample was filtered through a 0.45 μm membrane filter prior to HPLC analysis.

To obtain the single-analyte standard solutions, 6.25 mg of VNL, 5.00 mg of VN-2, 2.92 mg of VN-5, and 5.42 mg of VN-12 were weighed and dissolved with 10 mL of 50% v/v methanol. Then, a mixed standard solution containing 0.15 mg/mL VNL, 0.12 mg/mL VN-2, 0.07 mg/mL VN-5, and 0.13 mg/mL VN-12 was prepared. Standard solutions were prepared by serial dilution of the mixed solutions in 50% v/v



methanol to the working range of each substance for the establishment of calibration curves. All the standard solutions were stored at 4°C in the dark and brought to room temperature before analysis. The standard solutions were filtered through 0.45 µm membrane filters prior to HPLC analysis.

#### **2.4 Method validation of fingerprint analysis**

According to the guidelines of the SFDA of China [26], the developed HPLC-DAD fingerprinting method was validated in terms of its precision, stability, and repeatability.

#### **2.5 Method validation of quantitative analysis**

Sample No. S13 (collected from the Sichuan province) was used for method validation. Method precision was evaluated by seven consecutive injections of the same sample solution, whereas the repeatability was analyzed by seven replicates of the same powder sample. The stability of the sample solution was evaluated at the time points of 0, 3, 6, 9, 12, 24, 36 and 48 h, while the recovery was investigated by the standard addition method within the same day. Three different concentrations of mixed standard solutions were spiked into sample S13.

#### **2.6 Data Analysis**

The chromatographic profiles of all extracts were analyzed by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine software (Version 2004 A), which was recommended by the State Food and Drug Administration of China (SFDA) for calculating the correlation coefficients of chromatographic profiles of TCM [27]. The hierarchical cluster analysis (HCA) of all

the samples was performed using SPSS software (IBM SPSS Statistics 19, Armonk, New York, USA).

### **3 Results and discussion**

#### **3.1 Optimization of the chromatographic conditions**

To obtain chromatograms with better separation and sharper peaks, the chromatographic column, mobile phase, column temperature and detection wavelength were optimized.

Three reversed-phase columns, an Agilent SB-C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m), an Agilent Eclipse XDB-C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m) and an Agilent Zorbax Extend C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m), were used and compared. Among all the chromatographic columns, the Agilent Zorbax Extend C18 column gave better peak separation and sharper peaks.

The effect of the mobile phase composition (methanol/water, methanol/water/formic acid, acetonitrile/water, acetonitrile/water/formic acid and acetonitrile/methanol/formic acid) on the chromatographic separation was then assessed. As a result, acetonitrile and a 0.1% aqueous formic acid solution were chosen to achieve better selectivity and a higher efficiency.

In our experiment, six column temperatures—15, 20, 25, 30, 35 and 40°C—were chosen to examine their effect on the peak separation and the peak areas of the samples. The column temperature of 30°C afforded a better resolution and separation of the four markers within 70 min. Therefore, the optimal column temperature was determined to be 30°C, according to the data computed by the provided software

program.

With a UV full-scan of the four lignans in the range of 200 to 400 nm, maximum absorbance values were observed, and most of the characteristic peaks could be detected at approximately 254 nm. Thus, 254 nm was selected as the detection wavelength, and the reference wavelength was turned off. The optimal HPLC conditions used in this study are shown in Section 2.2.

### **3.2 Method validation of fingerprint analysis**

The precision of the HPLC fingerprint method was assessed by seven successive injections of the same sample (Sample No. S13) solution in a day. The correlation coefficients between the reference HPLC fingerprint and the sample HPLC fingerprint were no less than 0.992 ( $> 0.950$ ).

The sample stability test was evaluated by the same sample solution at different time intervals (0, 3, 6, 12, 24, 36 and 48 h) within 2 days. The correlation coefficients exceeded 0.995 ( $> 0.950$ ).

The repeatability was determined by analyzing seven independently prepared solutions of the same sample, and the correlation coefficients were greater than 0.990 ( $> 0.950$ ).

The results of the precision, stability and repeatability studies met the national standard for traditional Chinese medicine fingerprint analysis, [26] and the method was suitable for the fingerprint analysis of *V. negundo* seeds.

### **3.3 Method validation of quantitative analysis**

To further determine the contents of the four lignans, the analysis method was

validated by defining the linearity, limits of quantification and detection (LOD and LOQ), precision, repeatability, stability and recovery.

### **Linearity, LOD and LOQ**

Seven concentration gradients of standard compounds were injected in triplicate. The calibration curves were constructed by plotting the peak areas ( $y$ ) of the reference standards versus their concentrations ( $x$ , mg/L) under the selected optimal conditions. Good linearity ( $R^2 > 0.9995$ ) was observed within the tested concentration ranges for all target compounds. The limits of detection (LOD) and limits of quantification (LOQ) of four investigated compounds were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. A standard solution containing the four reference compounds was diluted to a series of appropriate concentrations with methanol. The diluted solutions were injected for analysis, and the results are shown in **Table 2**, which indicates that the analytical method and the HPLC apparatus are acceptable with sufficient sensitivity.

### **Precision, repeatability, stability and recovery tests**

The injection precision was evaluated by analyzing seven replicates of the sample solutions (Sample No. S13) continuously within one day based on the peak areas of the four reference lignans. The relative standard deviation (RSD) values of the peak areas of those four markers were found to be in the range of 0.191%-0.849%, indicating that the precision of the instrument was satisfactory.

The repeatability was assessed by analyzing seven individually prepared solutions of the same sample (Sample No. S13). The RSD values of the peak areas of the four

markers were less than 1.18%, which revealed the good reproducibility of the method.

The stability test was performed by analyzing the same sample solution (Sample No. S13) on two consecutive days at different time intervals (0, 3, 6, 12, 24, 36 and 48 h), and the RSD values of the peak areas of the four markers were below 1.43%, which indicated that the sample solution remained stable within 48 h.

The recovery rate was determined by the standard addition method. The sample (Sample No. S13) was spiked with three different concentration levels (50%, 100% and 150%) of the mixed standard solution of four lignans on a single day according to the established procedures (Section 2.1). The recoveries were 98.43-102.3%, and their RSD values were all less than 3%, which indicated that the HPLC-DAD method was accurate for the simultaneous quantitative analysis of those four lignans in *V. negundo* seeds.

The precision, repeatability, stability and recovery results are shown in **Table 2**, which indicates that our established HPLC method was precise, accurate and sensitive enough for the simultaneous quantitative study of those four lignans in *V. negundo* seeds.

### **3.4 Establishment of a chromatographic fingerprint of *V. negundo* seeds**

To standardize the HPLC profile, 13 *V. negundo* seed samples from different provinces of China were analyzed under the optimized HPLC conditions, and all the chromatograms were matched by the computer-aided *Similarity Evaluation System for Chromatographic Fingerprint of TCM* (Version 2004A) (**Fig. 2**). Peaks shared by all the chromatograms of the tested samples were assigned as “characteristic common

peaks” to represent the characteristics of all the samples. A total of 21 common peaks (from peak 1 to 21) were found in the chromatogram, which covered more than 80% of the total area (**Fig. 3**). Four peaks were identified as 6-hydroxy-4 $\beta$ -(4-hydroxy-3-methoxyphenyl)-3 $\alpha$ -hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde (VNL) (peak 12), vitedoin A (VN-2) (peak 13), vitedoamine A (VN-5) (peak 14), and vitexdoin A (VN-12) (peak 7) by comparing their retention times and UV spectra with those of standard compounds. Due to the moderate retention time, height and good shape of peak 12 (VNL) in the fingerprint, this peak can be assigned as the reference peak. It was thus convenient to calculate the relative retention time (RRT) and relative peak area (RPA) of each characteristic peak compared with peak 12, known as VNL, which has also been proven to be one of the most important active constituents of *V. negundo* seeds [9, 16]. The RRTs and RPA data of these 21 peaks were thus calculated based on this reference peak (peak 12). The results are listed in **Table 3**. The RRT and RPA data of the characteristic peaks could be used to monitor the internal quality of *V. negundo* seeds.

### **3.5 Similarity analysis of the HPLC fingerprints of *V. negundo* seeds**

The similarity values of all the samples were calculated using the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A). Similarities were generated by comparing the 13 *V. negundo* seed samples with the standard chromatogram (R as shown in **Fig. 3**), and the similarity values are presented in **Table 1**, ranging from 0.647 to 0.971. Samples S1, S3, S4, S6 and S11 displayed relatively low similarity, with similarity values of 0.887, 0.833, 0.861, 0.647 and

0.857, respectively, while the similarity values of the other samples were all higher than 0.902. From the chromatograms, we can find that the contents of VNL and VN-2 in S3 and S6 are very low compared with the average value. Samples S1, S3, S4 and S11 had many chromatographic peaks in the range of 26-40 min, indicating the presence of abundant moderately-polar constituents. The types and quantities of variations in the chemical components would absolutely cause the decrease in similarity. Samples S1, S3, S4, S6 and S11 were all collected in local mountain areas, whereas most of the other samples were collected in brushwood near streams. The varied chemical profiles of *V. negundo* seeds may be attributed to the different geographic locations, climate conditions and habitats.

### **3.6 Quantitative determination of four lignans in *V. negundo* seeds**

In this study, the method developed above was successfully applied to the simultaneous determination of four major lignans in 13 *V. negundo* seed samples. The HPLC chromatogram of these four lignans is shown in **Fig. 4**. The peaks of VNL, VN-2, VN-5 and VN-12 in each sample are identified by comparing the retention times and the UV spectra with those of the standards. Quantification was determined by the external standard method using calibration curves fitted by regression equations. The analyses were performed in triplicate, and the results are shown in **Table 1**.

The contents of the four lignans in the 13 *V. negundo* seed samples varied significantly. Our results show that VNL and VN-2 are two major lignans found in the seed samples, with ranges of 0.46-3.20 mg/g and 0.40-2.27 mg/g, respectively. The

largest variation was found in the content of VN-5 with an RSD of 140.93% in the 13 samples, indicating that the content of VN-5 in the samples from different regions was evidently different. Moreover, VN-5 can hardly be detected in several samples. Considering the notable variation in the lignan contents of *V. negundo* seeds collected from different locations and the validated bioactivities of VNL and VN-2, we suggest that just VNL and VN-2 should be used as the indicator compounds to characterize the quality of these medicinal seeds.

### 3.7 Hierarchical Clustering Analysis (HCA)

HCA, one of the chemical pattern recognition and classification evaluation methods, is used to set the level of bottom-up decomposition for a given data set until certain conditions are fulfilled. HCA has been commonly applied for fingerprint analysis with standard normal variant transformation of the data, which led to meaningful classification of herbal samples collected from different regions [27]. The hierarchical cluster analysis in this study was performed based on the relative peak areas of those common characteristic peaks (peak 1-21) calculated by the Similarity Evaluation System.

To assess the similarity and differences between various samples, Q-cluster analysis was applied to sort the 13 *V. negundo* seed samples into groups. A dendrogram of HCA is shown in **Fig. 5**, which shows that the 13 samples are classified into three quality clusters. Among them, samples S2, S5, S7, S9, S10, S12 and S13 are in Cluster A, whereas Cluster B includes the samples S1, S3, S4 and S8, and the others are in Cluster C. Compared with the chemotypes of these samples (**Fig.**



2), the sorting of the 13 samples is consistent with the categorized results of their apparent chromatograms. This HCA result may provide more references for further quality studies of this Chinese herbal medicine.

#### **4 Conclusion**

In the present study, a HPLC method combined with fingerprint analysis and the simultaneous determination of four bioactive lignans was first developed to evaluate the quality of *V. negundo* seeds, with high precision, stability, and repeatability. A total of 13 samples collected from different regions of China were assessed by chromatographic fingerprint and hierarchical cluster analysis. The similarities of the 13 samples ranged from 0.647 to 0.971 based on 21 characteristic fingerprint peaks, indicating that the quality of *V. negundo* seeds can be affected by different plant habitats. Four lignans in the 13 *V. negundo* seed samples were successfully separated and quantitatively determined, which showed that the contents of the four lignans in *V. negundo* seeds displayed notable differences in herbal samples collected from different regions. The HCA method clustered the samples into three groups, which were in good agreement with the visual comparison of their chemotype chromatograms. Therefore, the rapid and effective method developed in our study, although simple and plain, provided a solid foundation for establishing reasonable quality control standards for *V. negundo* seeds. Our work also demonstrated that the HPLC combination of fingerprint and quantitative analysis of active components is a powerful and practical tool for comprehensive quality assessment of TCM.

#### **Acknowledgements**

*This work was supported by the National Natural Science Foundation of China (Nos. 81102773; 81473328), the Outstanding Youth Program of Shanghai Medical System (XYQ2013100), the Innovation Plan of Science and Technology Commission of Shanghai Municipality (14401902500) and the Postdoctoral Program of Science and Technology Commission of Shanghai Municipality (14R21411600).*

**Conflict of interest**

*The authors have declared no conflict of interest.*

## 5 References

- [1] Wang, L., Yuan, K., Yu, W. W., *Pharmacogn. Mag.* 2010, 6, 83-88.
- [2] Gao, H. M., Wang, Z. M., Li, Y. J., Qian, Z. Z., *Front. Med.* 2011, 5, 195-202.
- [3] Liang, Y. Z., Xie, P. S., Chan, K., *J. Chromatogr. B* 2004, 812, 53-70.
- [4] Liang, X. R., Ma, M. L., Su, W. K., *Pharmacogn. Mag.* 2013, 9, 238-243.
- [5] Qin, K. M., Wang, B., Li, W. D., Cai, H., Chen, D. N., Liu, X., Yin, F. Z., Cai, B. C., *J. Sep. Sci.* 2015, 38, 1491-1498.
- [6] Yao, W. F., Dai, J., Zheng, C. Z., Bao, B. H., Cheng, H. B., Zhang, L., Ding, A. W., Li, W., *J. Sep. Sci.* 2015, 38, 1822-1827.
- [7] Jiang, Y., David, B., Tu, P. F., Barbin, Y., *Anal. Chim. Acta.* 2010, 657, 9-18.
- [8] Tang, D. Q., Li, Z., Jiang, X. L., Li, Y. J., Du, Q., Yang, D. Z., *J. Sep. Sci.* 2014, 37, 2131-2137.
- [9] Zheng, C. J., Huang, B. K., Han, T., Zhang, Q. Y., Zhang, H., Rahman, K., Qin, L. P., *J. Nat. Prod.* 2009, 72, 1627-1630.
- [10] Vimal, A., Vikram, L., Singhal, S., Anil, B., *Int. J. Pharm. Innov.* 2011, 1, 9-20.
- [11] Zheng, C. J., Zhao, X. X., Ai, H. W., Lin, B., Han, T., Jiang, Y. P., Xing, X., Qin, L. P., *Phytomedicine* 2014, 21, 838-846.
- [12] Bhargava, S. K., *J. Ethnopharmacol.* 1989, 27, 327-339.
- [13] Ono, M., Nishida, Y., Masuoka, C., Li, J. C., Okawa, M., Ikeda, T., Nohara, T., *J. Nat. Prod.* 2004, 67, 2073-2075.
- [14] Zheng, C. J., Huang, B. K., Wang, Y., Ye, Q., Han, T., Zhang, Q. Y., Zhang, H., Qin, L. P., *Bioorgan. Med. Chem.* 2010, 18, 175-181.
- [15] Zheng, C. J., Li, H. Q., Ren, S. C., Xu, C. L., Rahman, K., Qin, L. P., Sun, Y. H., *Phytother. Res.* 2015, 29, 633-647.
- [16] Zhou, Y. J., Liu, Y. E., Cao, J. G., Zeng, G. Y., Shen, C., Li, Y. L., Zhou, M. C., Chen, Y. D., Pu, W. P., Potters, L., *Clin. Cancer Res.* 2009, 15, 5161-5169.
- [17] Xin, H., Kong, Y., Wang, Y., Zhou, Y. J., Zhu, Y. Z., Li, D. P., Tan, W. F., *Phytomedicine* 2013, 20, 640-647.
- [18] Hu, H. Y., Zhou, J., Wan, F., Dong, L. F., Zhang, F., Wang, Y. K., Chen, F. F., Chen, Y. D., *J. Zhejiang Univ.* 2012, 41, 498-505.
- [19] Cai, Y. L., Chen, Z. D., Tang, A. Q., *J. Nanhua Univ.* 2011, 39, 153-155.
- [20] Zheng, C. J., Zhang, X. W., Han, T., Jiang, Y. P., Tang, J. Y., Brömme, D., Qin, L. P., *Fitoterapia* 2014, 93, 31-38.
- [21] Zheng, C. J., Lan, X. P., Cheng, R. B., Huang, B. K., Han, T., Zhang, Q. Y., Zhang, H., Rahman, K., Qin, L. P., *Phytochem. Lett.* 2011, 4, 298-300.
- [22] Cai, Y. L., Zhou, Y. J., Xiang, H. L., Cao, J. G., Xie, W. Y., *J. Hunan Normal Univ.* 2008, 5, 24-28.
- [23] Zheng, G. M., Li, Z. J., Liu, G. Y., Fu, Z., Zhang, X. C., *Fine Chemicals* 2012, 29, 366-368.
- [24] Zheng, C. J., Tang, W. Z., Huang, B. K., Han, T., Zhang, Q. Y., Zhang, H., Qin, L. P., *Phytomedicine* 2009, 16, 560-567.
- [25] Xu, C. J., Liang, Y. Z., Chau, F. T., Heyden, V. Y., *J. Chromatogr. A* 2006, 1134, 253-259.
- [26] Drug Administration Bureau of China, Requirements for Studying Fingerprint of Traditional Chinese Medicine Injections (Draft), 2000.

[27] Wu, Y. B., Zheng, L. J., Yi, J., Wu, J. G., Chen, T. Q., Wu, J. Z., *Int. J. Mol. Sci.* 2013, 14, 1999-2010.

## Figures and Tables

**Figure 1.** Chemical structures of VNL, vitedoin A, vitedoamine A, and vitexdoin A

(VNL:

6-hydroxy-4 $\beta$ -(4-hydroxy-3-methoxyphenyl)-3 $\alpha$ -hydroxymethyl-7-methoxy-3,4-dihy

dro-2-naphthaldehyde; VN-2: vitedoin A; VN-5: vitedoamine A; and VN-12:

vitexdoin A)

**Figure 2.** Chromatographic fingerprints of 13 *Vitex negundo* seed samples showing

three chemotypes. The denotations from S1 to S13 are the corresponding sample

numbers as listed in Table 1. The separation conditions are described in Section 2.2.

**Figure 3.** The reference fingerprint of *Vitex negundo* seeds showing 21 common

peaks originating from the Similarity Evaluation System for Chromatographic

Fingerprint of TCM software (Version 2004A).

**Figure 4.** HPLC chromatographic profile of four mixed standards (VNL, Peak 12;

VN-2, Peak 13; VN-5, Peak 14; and VN-12, Peak 7).

**Figure 5.** Dendrograms of hierarchical cluster analysis of 13 *Vitex negundo* seed

samples. The denotations from S1 to S13 are the corresponding sample numbers as

listed in Table 1 (dendrograms using average linkage between groups).

**Table 1.** Contents (mg/g) of four lignans in 13 *Vitex negundo* seed samples and their

fingerprint similarity.

**Table 2.** Linear ranges, regression equations,  $R^2$  values, LODs, LOQs, precisions,

stabilities, repeatabilities, and recoveries of the four lignans (n = 7).

**Table 3.** The retention time ( $t_R$ ), relative retention time (RRT), peak area (PA) and

relative peak area (RPA) values of 21 characteristic fingerprint peaks in *Vitex negundo* seeds.