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# Molecular Genetic and Chemical Assessment of Radix *Angelica* (Danggui) in China

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The roots of *Angelica sinensis* (Danggui), a traditional Chinese medicine, have been used for invigorating blood circulation for over 2000 years in China. Three common species of *Angelica* roots are found in Asia: *A. sinensis* from China, *A. acutiloba* from Japan, and *A. gigas* from Korea. By using a molecular genetic approach, the 5S-rRNA spacer domains of the three species of *Angelica* were amplified, and their nucleotide sequences were determined. Diversity in DNA sequences among various species was found in their 5S-rRNA spacer domains, which could serve as markers for authentic identification of *Angelica* roots. In chemical analyses, the main constituents of *Angelica* roots including ferulic acid and *Z*-ligustilide were determined by HPLC; roots of *A. sinensis* were clearly distinct in that they contained ~10-fold higher levels of ferulic acid and *Z*-ligustilide as compared to roots of *A. acutiloba* and *A. gigas*. In addition, the amounts of main constituents in roots of *A. sinensis* varied according to different regions of cultivation and different methods of preservation. The chemical profile determined by HPLC therefore could serve as a fingerprint for quality control of *Angelica* roots.

**KEYWORDS:** ***Angelica*; authentic identification; HPLC analysis; TCM; 5S-rRNA spacer domains**

## INTRODUCTION

Radix Angelica (roots of *Angelica*; Danggui) is a common traditional Chinese medicine that has been used for more than 2000 years in China. The use of Radix Angelica was first recorded in *Shen Nong Bencao Jing* in ~100 B.C.; the described functions were to replenish blood, to invigorate blood, to stop pain, and to moisten the intestines. Nowadays, it is used to invigorate blood circulation for the treatment of menstrual disorders (*1*−*3*), to modulate the immune system (*4*), and as an emollient and laxative for chronic constipation of the aged and debilitated (*5*, *6*). The Chinese Pharmacopoeia (2000) recorded 70 formulas containing *Angelica* roots in China, and 56 formulas containing *Angelica* roots in Japan (*7*). Besides the common usage in Asia, *Angelica* root is also used as a health food product for women's care in Europe and America. Therefore, the demand for Chinese *Angelica* root is enormous throughout the world (*8*). The Chinese Pharmacopoeia (2000) recorded that *Angelica* root in China is derived from the root of *Angelica sinensis* (Oliv.) Diels (family Umbellaceae) (*9*). However, *Angelica acutiloba* (Sieb. et Zucc.) Kitag. is mainly found in Japan, and *Angelica gigas* Nakai is mainly found in Korea; these two species are commonly found in the markets of Southeast Asia, which could serve as substitutes of Radix Angelica due to the shortage of *A. sinensis* (*10*−*13*). The three commonly used *Angelica* roots showed variation in their chemical compositions, pharmacological properties, and efficacies; these problems in quality control, therefore, compromise the values of traditional Chinese medicine or even jeopardize the safety of the consumers (*10*).

The main chemical constituents of *Angelica* roots are ferulic acid, ligustilide, angelicide, brefeldin A, butylidenephthalide, butyphthalide, succinic acid, nicotinic acid, uracil, and adenine (*14*−*16*). The constituents most often associated with the pharmacological activities of *Angelica* roots are ferulic acid and ligustilide (predominantly the *Z*-isomer), the former being able to inhibit platelet aggregation and serotonin release, the latter having significant antiasthmatic and spasmolytic activities (*14*). The levels of these two constituents are usually used as chemical markers for the quality control of *Angelica* roots (*15*, *16*). Besides the variation of chemical composition among different species of *Angelica*, the amounts of ferulic acid and *Z*-ligustilide within *Angelica* roots could vary significantly according to their geographical sources. *A. sinensis* is mainly cultivated in Gansu province and partly in Yunnan, Sichuan, and Shanxi provinces of China, whereas *A. acutiloba* and *A. gigas* are distributed

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mainly in Japan and Korea with only a very small quantity found in China, including the Jilin, Liaoning, and Yunnan provinces. Su Jing (659 A.D.) in *Tang Ben Cao* and Li Shi Zhen (1596 A.D.) in *Ben Cao Gang Mu* reported that *Angelica* root produced in Gansu, China, was the authentic source and had the superior quality.

To confirm the quality control of *Angelica* roots, we determined the genetic and chemical distinction of roots derived from *A. sinensis*, *A. acutiloba*, and *A. gigas*. The 5S-rRNA spacer regions, unique intronic sequences that have been used commonly for species authentication (*17*−*20*), were sequenced and compared among different species of *Angelica*. Chemically, we compared the contents of ferulic acid and *Z*-ligustilide in roots of *A. sinensis*, *A. acutiloba*, and *A. gigas*, as well as their variation according to different cultivated regions in China.

## MATERIALS AND METHODS

**Plant Materials.** Fresh plants were obtained from China: *A. sinensis* from Minxian of Gansu, Lijiang of Yunnan, Nanping of Sichuan, and Pingli of Shanxi collected by ourselves; *A. acutiloba* from Japan collected by Dr. Hui Y. Li of National Research Institute for Traditional Sino-Japanase Medicines, Toyama Medical and Pharmaceutical University; *A. gigas* from Korea collected by Dr. Xiu H. Ji of National Products Chemistry Laboratory, Department of Applied Biological and Environmental Chemistry, Seoul National University; and *Angelica dahurica* (Fisch. ex Hoffm.) Benth et Hook (Baizhi), *Angelica pubescens* Maxim. (Duhuo), and *Ligusticum chuanxiong* Hort. (Chuanxiong) from Sichuan.

All of the plant materials were collected in September or October after they had been cultivated for 2 years. The botanical origins of all the materials in forms of whole plants were identified morphologically by ourselves during the field collection in different regions of China. Different *Angelica* species from geographical properties of the same region were collected and dried under vacuum. The chosen area was based on the abundance of Radix Angelica that was found. About 10 batches of individual species having similar but not identical geographical properties of the same region were tested. Individual samples were prepared from ∼500 g of powder that was ground from ∼20 plants of the same population. These grinding processes were done during the field collection before they were delivered to the laboratory. The collected powder was stored with silica gel, which stabilized the chemical constituents. Their corresponding voucher specimens as forms of whole plants were deposited with the Department of Biology, The Hong Kong University of Science and Technology, Hong Kong, China.

Morphological differences of the three species of *Angelica* plants were based on the following characterizations (*15*): *A. sinensis* contains a once, doubly, or triply ternate-pinnate leaf, a compound umbel with 9−13 small umbels, cremocarp, and in the commissural surface 2 vittae occur; *A. acutiloba* contains a once or doubly ternate-pinnate leaf, a compound umbel with ∼22 small umbels, cremocarp, and in the commissural surface 8 vittae occur; *A. gigas* contains a once or doubly ternate-pinnate leaf, a compound umbel with ∼30−70 small umbels, cremocarp, and in the commissural surface 2 vittae occur.

**DNA Extraction.** Genomic DNA was extracted from the ground powder derived from crude herbs by using a DNA extraction buffer consisting of 25 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.5% SDS, 10 $\mu$g/mL RNase, and 0.2% $\beta$-mercaptoethanol. The mixed solution was incubated for 15 min at 58 °C and then centrifuged at 12000*g* for 10 min. The supernatant was extracted by an equal volume of water-saturated phenol/chloroform (1:1), mixed, and then centrifuged. The aqueous phase was collected and added to 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. The resulting pellet was collected after centrifugation and dissolved in 10 mM Tris-HCl, pH 8.0, and 5 mM EDTA (Tris-EDTA buffer).

**PCR Amplification and Sequencing.** A 50 $\mu$L PCR reaction mixture consisted of 5 $\mu$L of 10× reaction buffer, 1 $\mu$L each of 10 mM dNTPs stock, 2.5 $\mu$L containing 0.3 $\mu$g of forward and reverse primers (synthesized by Invitrogen Life Technologies, Carlsbad, CA), and 1 unit of *Taq* polymerase (Roche, Mannheim, Germany). The primers used for amplification of 5S-rRNA were 5S-P1 forward primer (5′-GGA TTC GTG CTT GGG CGA GAG TAG TA-3′) and 5S-P2 reverse primer (5′-ACG CTA GTA TGG TCG TGA TTC CTA GG-3′). These primers flank the spacer domain of 5S-rRNA (*18*, *21*, *22*). Approximately 50 ng of genomic DNAs was used as a template for the reaction. The reaction mixture was overlaid with mineral oil and placed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA). Cycling conditions consisted of an initial 5 min at 94 °C followed by 1 min of denaturing at 94 °C, 2 min of annealing at 53 °C, and 3 min of elongation at 72 °C repeated for 30 cycles with a 10 min extension at 72 °C. The PCR products were subjected to ∼1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV. DNAs were purified by a Concert Rapid gel extraction system (Invitrogen Life Technologies).

The PCR products were subcloned into a TA cloning vector pTAg (Promega, Madison, WI). Competent *Escherichia coli* JM 109 cells were transformed with the ligation products, and the colonies identified by color selection were picked and grown in 3 mL of Luria−Bertani (LB) liquid medium overnight. The minipreparation of plasmid DNAs from the ligator cell was performed using alkaline lysis (*23*). The correct DNA inserts were verified by restriction analysis, and the plasmid DNAs, verified by colonies, were isolated with the Concert nucleic acid purification system (Invitrogen Life Technologies). An ABI Prism, a BigDye Terminator, and a cycle Sequencing Ready Reaction kit were used for sequencing reaction with T7 or SP6 primers. Sequences were detected by an ABI Prism 310 genetic analyzer (Applied Biosystems). Both strands of DNA clones were sequenced at least twice, and the sequences were aligned by MacVector software (Kodak, New Haven, CT). Comparisons of entire sequences of test samples were aligned by software Clustal X software having a gap opening of 10 and a gap extension of 0.05. A polygenetic tree based on the hierarchical clustering of the alignments of the 5S-rRNA spacers was assessed by the neighbor-joining (UPGMA) method.

**Extraction of Chemical Constituents.** About 0.5 g of ground powder was soaked overnight in 10 mL of aqueous methanol and then extracted under ultrasonic conditions for 30 min twice. The methanol extracts were combined and centrifuged at 3500 rpm for 20 min. The supernatant was collected and filtered through a Millipore filter unit. Twenty microliters of the sample was injected to reverse phase high-performance liquid chromatography (HPLC).

**Quantitative Analysis of Ferulic Acid and *Z*-Ligustilide.** Ferulic acid was purchased from Sigma (St. Louis, MO), and *Z*-ligustilide was purified within our laboratory and identified by mass spectrometry spectrum and nuclear magnetic resonance. HPLC grade reagents were from Fischer and Labscan (Dublin, Ireland). Standards of ferulic acid (1 mg) and *Z*-ligustilide (2 mg) were weighed and dissolved in 1 mL of methanol to give serial concentrations, and three injections were performed for each dilution. The standard curve was calibrated by using the linear least-squares regression equation derived from the peak area. The concentrations of these two compounds in the samples were calculated according to the regression parameters derived from the standard curves. HPLC was performed in a reverse phase Nova-Pak $C_{18}$ column (150 mm × 3.9 mm i.d., particle size = 4 $\mu$m) with a guard column (Nova-Pak $C_{18}$, 20 mm × 3.9 mm i.d., particle size = 4 $\mu$m), in a Waters PC 800 integrator, a Waters 486 tunable absorbance detector, and a Waters 600 pump. Chromatography was carried out with a linear gradient program. Solvents were (A) water (pH 4.0 adjusted with phosphoric acid) and (B) acetonitrile. The gradient of A:B was 0−10 min, 95:5 gradient up to 65:35; 10−30 min, gradient up to 30:70; 30−40 min, 95:5. The flow rate of mobile phase was kept constant at 1.0 mL/min. Peaks were detected at 284 nm. In hierarchical clustering analysis of the different samples, SPSS software (version 11.0.0 from Statistical Product and Service Solutions, Chicago, IL) was used.

## RESULTS AND DISCUSSION

Primers flanking the spacer domain of 5S-rRNA were used in PCR analysis of genomic DNAs isolated from *A. acutiloba*, *A. gigas*, and four geographic strains of *A. sinensis* including Minxian of Gansu, Lijiang of Yunnan, Nanping of Sichuan, and

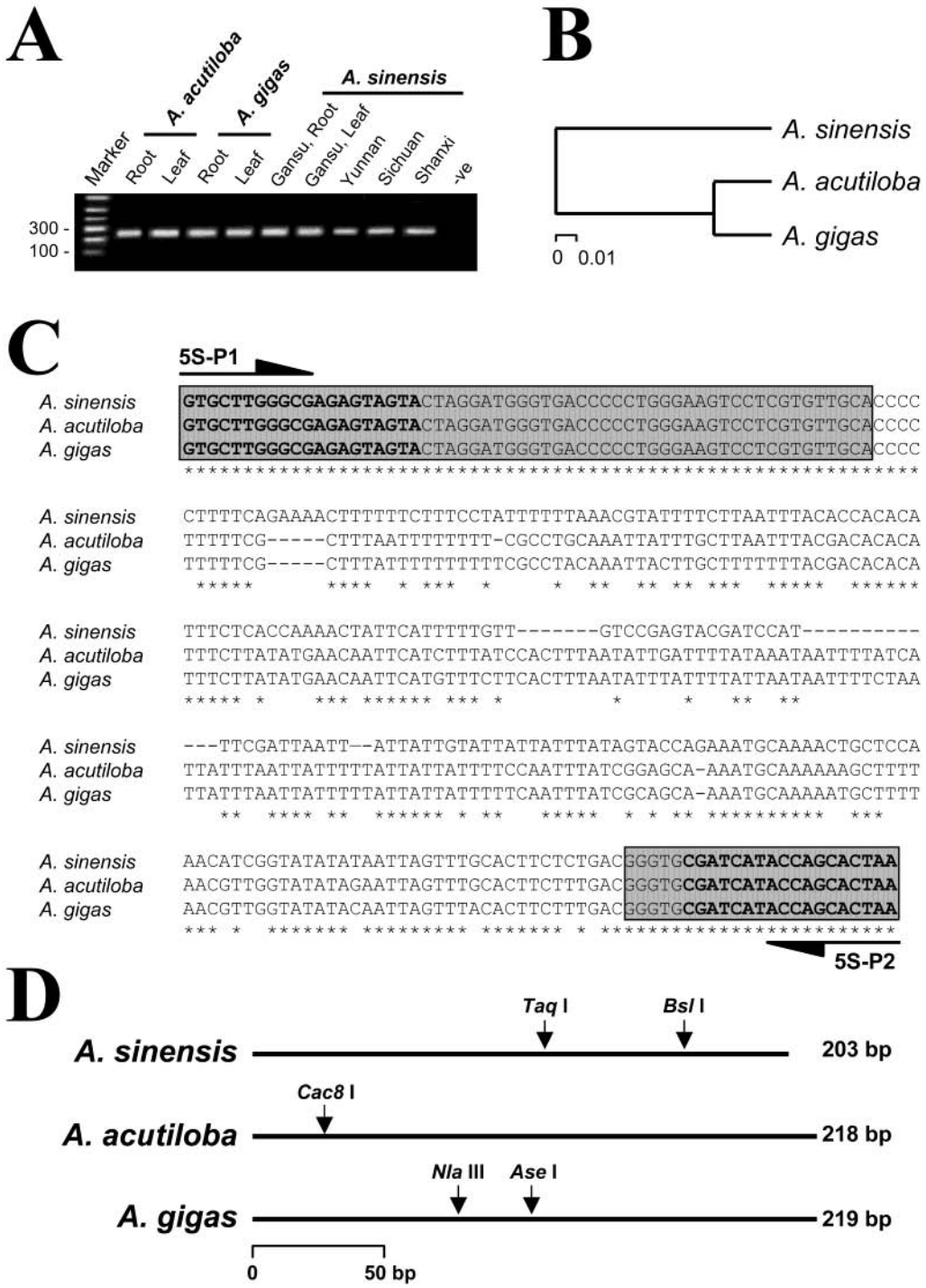


**Figure 1.** Genetic analyses of different species of *Angelica*: (A) Primers (5S-P1 and 5S-P2) flanking the spacer domain of the 5S-rRNA spacer domain were used for PCR analysis, and the PCR products were separated by a 1% agarose gel and visualized by ethidium bromide staining. PCR products having similar sizes were obtained from five individual samples ($n = 5$). The same PCR product was obtained either from crude drugs (root) or fresh materials (leaf) as well as four geographic strains including Minxian of Gansu, Lijiang of Yunnan, Nanping of Sichuan, and Pingli of Shanxi; *A. acutiloba* and *A. gigas* were collected from Japan and Korea. The negative control is the one without genomic DNA. DNA markers in bp are indicated. (B) A phylogenetic tree is shown for *Angelica* species assessed by the UPGMA method. *A. acutiloba* and *A. gigas* show great DNA diversity when compared to *A. sinensis*. The distance corresponding to 0.01 sequence divergence is indicated by the bar. (C) Sequence alignments of the spacer domains of 5S-rRNA from *A. sineness* (GenBank accession no. AY185119), *A. acutiloba* (GenBank accession no. D63407), and *A. gigas* (GenBank accession no. AY185120) are shown. The coding regions are boxed. Primer sequences (5S-P1 and 5S-P2) are indicated by arrows. Identical sequences are indicated by an asterisk (∗). Caps (–) are introduced for the best alignment. (D) Restriction enzyme mapping of 5S-rRNA spacer domains of *A. sinensis*, *A. acutiloba*, and *A. gigas* is shown. Restriction sites for *Taq*I, *Bsl*I, *Cac*8I, *Nla*III, and *Ase*I are shown by arrows. Only the distinct sites are shown here, which could be used for quick authentic identification.

Pingli of Shanxi. The major PCR products of the tested species were ~300 bp in the electrophoresis analysis (**Figure 1A**). The PCR products were subcloned and sequenced. Several individual clones of the same PCR product were sequenced to avoid any

mutation introduced by *Taq* polymerase. The respective lengths of the 5S-rRNA spacer domains generated by PCR in different species are as follows: *A. sinensis*, 296 bp; *A. acutiloba*, 311 bp; and *A. gigas*, 312 bp (**Figure 1C**). Identical sequences were obtained when the amplification was carried out on DNAs isolated either from fresh leaves or dry crude herbs of the same species. In addition, no difference in the 5S-rRNA spacer sequence was found among the four geographic strains of *A. sinensis* (data not shown). The sequence of *A. sinensis* is 72.87% and 73.58% DNA identical to those of *A. acutiloba* and *A. gigas*, respectively, whereas *A. acutiloba* and *A. gigas* share DNA identity of 93.57% in their sequences (**Figure 1C**). From the restriction enzyme mapping of the sequenced spacer domains, *Taq*I and *Bsl*I sites are present within the spacer domain of *A. sinensis* but not in those of *A. acutiloba* and *A. gigas*, which provides a rapid authentication of *Angelica* species (**Figure 1D**). The phylogenetic tree based on the sequences of 5S-rRNA spacer domains by using UPGMA method is shown in **Figure 1B**. The result clearly revealed that the three *Angelica* species are phylogenetically divided into two clusters: the first group consisting of *A. sinensis* and the second group consisting of *A. acutiloba* and *A. gigas*.

The HPLC calibration curves of ferulic acid and *Z*-ligustilide exhibited good linearity in a range from 0.1 to ~12.8 $\mu$g/mL. The coefficients of correlation were 0.9994 (ferulic acid) and 0.9996 (*Z*-ligustilide). The precision test within the same sample of ferulic acid and *Z*-ligustilide ($n = 8$) showed the relative standard deviation (RSD) values of 1.36% (ferulic acid) and 1.34% (*Z*-ligustilide). The repeatability of the constituents was excellent, having RSDs of 0.77% (ferulic acid) and 1.20% (*Z*-ligustilide). The recovery experiment was carried out to evaluate the accuracy of the method. Known amounts of ferulic acid and *Z*-ligustilide were added to the sample and extracted accordingly; the extracted material was subjected to analysis, and the contents of these two compounds were calibrated. The average recoveries of the tested ferulic acid and *Z*-ligustilide were 97.83 and 97.82% ($n = 5$), respectively. Three different extraction methods for determining the contents of ferulic acid and *Z*-ligustilide were calibrated: (i) the powder was extracted by methanol reflux; (ii) the powder was extracted by methanol filtration; and (iii) the powder was extracted by methanol on ultrasonic. The results suggested that method iii was the best because of the higher contents and good separation of ferulic acid and Z-ligustilide. **Figure 2A** shows typical chromatograms of methanol extracts of roots from *A. sinensis* (from Gansu), *A. acutiloba* (from Japan), and *A. gigas* (from Korea). The peaks of ferulic acid and *Z*-ligustilide were rather distinct, which were further identified by two means: (i) by comparing the retention times of the unknown peaks with those of the standards eluted under the same conditions and (ii) by spiking the sample with stock standard solutions of ferulic acid and *Z*-ligustilide (data not shown). In general, *Angelica* roots contains ~30% of total volatile oils as *Z*-ligustilide; on the other hand, the stereoisomer *E*-ligustilide could also be found in *Angelica* roots, but it contributes <1% of total volatile oils (*15*). Moreover, ferulic acid is being analyzed instead of its common ester form coniferyl ferulate, which exists in *Angelica* roots in a range from 0.06 to 0.11 wt %; this concentration range, in many circumstances, is in line to with of ferulic acid (from 0.04 to 0.12%) (*24*). Ferulic acid is generally recognized as an active constituent of *Angelica* roots (*6*, *15*). Thus, the contents of ferulic acid and *Z*-ligustilide in roots of *A. sinensis* from Gansu were ~10-fold higher than in roots derived from *A. acutiloba* (from Japan) and *A. gigas* (from Korea) (**Figure 2B**).

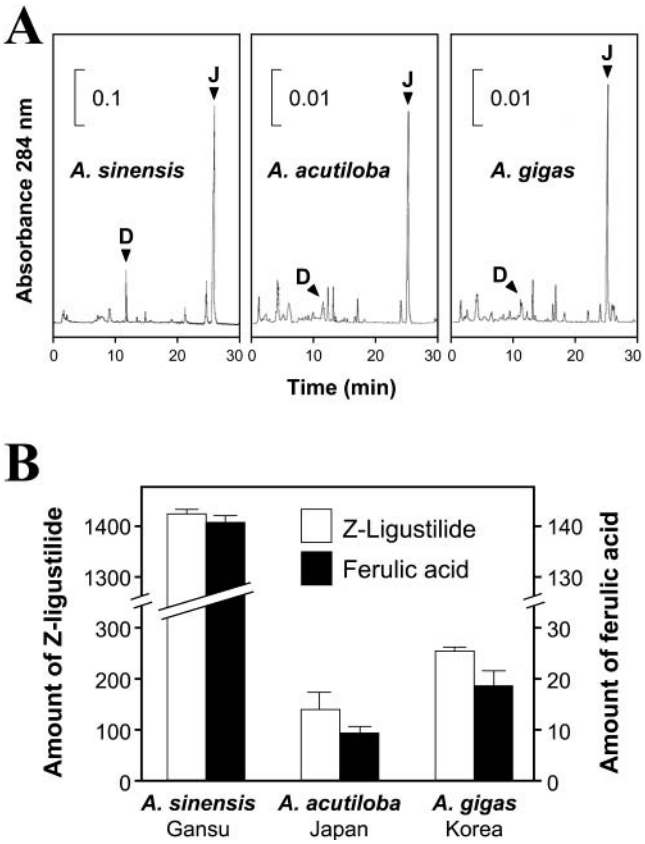


**Figure 2.** Determination of the contents of ferulic acid and *Z*-ligustilide from different species of *Angelica* roots: (A) typical HPLC chromatograms of methanol extracts from different *Angelica* roots [peaks corresponding to ferulic acid (D) and *Z*-ligustilide (J) are indicated; scale bars indicate absorbance at 284 nm]; (B) histogram showing the amount of ferulic acid and *Z*-ligustilide, determined as in (A), from roots of *A. sinensis* from Minxian of Gansu, *A. acutiloba* from Hokkaido, Japan, and *A. gigas* from Sokcho, Korea. The amounts of ferulic acid and *Z*-ligustilide are in mg/100 g of dried roots. Values are means ± SD, $n = 10$.

Historically, Gansu province is known to produce the best Radix Angelica in China; however, the variation within the subregions of Gansu provinces has not been determined. The contents of ferulic acid and *Z*-ligustilide were determined by HPLC from roots of *A. sinensis* derived from different regions of Gansu and other provinces of China. Thirteen different populations of *Angelica* roots from various geographical regions were collected; each chosen population contained 10−15 different batches of samples. Despite different subregions, Gansu contained a ~2-fold higher amount of *Z*-ligustilide and ferulic acid when compared with *Angelica* roots cultivated in Yunnan, Shanxi, or Sichuan (**Table 1**). Within different subregions of Gansu, Xizai of Minxian showed the best quality of *Angelica* roots in terms of the contents of *Z*-ligustilide and ferulic acid; however, the values were just slightly higher than those from other parts of Minxian in Gansu. **Table 1** also shows that there are very small variations with regard to the contents of *Z*-ligustilide and ferulic acid from different subregions of other provinces of China. Similarly, roots from *A. acutiloba* (from Japan) and *A. gigas* (from Korea) shared a close chemical resemblance even though they were obtained from two different subregions. This result contradicts a previous report that roots of *A. gigas* did not contain any *Z*-ligustilide (*25*). In addition, these results suggest that it is inappropriate to use roots from *A. acutiloba* and *A. gigas* as substitutes for *A. sinensis* roots. Although roots of *A. sinensis* from the Gansu area contain higher

**Table 1.** Contents of Ferulic Acid and *Z*-Ligustilide from Different Populations of Radix *Angelica*

| population no.[b] | species | voucher no. | cultivation region[c] | content[a] (mg/100 g) ferulic acid | content[a] (mg/100 g) *Z*-ligustilide |
|---|---|---|---|---|---|
| 1 | *A. sinensis* | 01-10-01 | Hadab, Dangchang, Gansu | 87 ± 0.70 | 941 ± 11.29 |
| 2 | *A. sinensis* | 01-10-02 | Meichuan, Minxian, Gansu | 90 ± 0.69 | 1107 ± 13.28 |
| 3 | *A. sinensis* | 01-10-05 | Shili, Minxian, Gansu | 65 ± 0.50 | 1138 ± 13.66 |
| 4 | *A. sinensis* | 01-10-08 | Xijiang, Minxian, Gansu | 62 ± 0.48 | 990 ± 11.88 |
| 5 | *A. sinensis* | 01-10-10 | Zhongzai, Minxian, Gansu | 109 ± 0.84 | 1138 ± 13.66 |
| 6 | *A. sinensis* | 01-10-12 | Qingshui, Minxian, Gansu | 86 ± 0.66 | 1013 ± 12.16 |
| 7 | *A. sinensis* | 01-10-13 | Xizai, Minxian, Gansu | 143 ± 1.10 | 1460 ± 17.52 |
| 8 | *A. sinensis* | 01-09-12 | Lijiang, Yunnan | 59 ± 0.45 | 563 ± 6.76 |
| 9 | *A. sinensis* | 01-10-15 | Zhongdian, Yunnan | 54 ± 0.42 | 547 ± 6.56 |
| 10 | *A. sinensis* | 01-09-04 | Baoxing, Sichuan | 40 ± 0.31 | 487 ± 5.84 |
| 11 | *A. sinensis* | 01-09-03 | Nanping, Sichuan | 44 ± 0.34 | 403 ± 4.84 |
| 12 | *A. sinensis* | 01-09-01 | Longxian, Shanxi | 35 ± 0.27 | 491 ± 5.89 |
| 13 | *A. sinensis* | 01-09-02 | Pingli, Shanxi | 38 ± 0.29 | 454 ± 5.45 |
| 14 | *A. acutiloba* | 01-09-25 | Toyama, Japan | 9 ± 0.07 | 149 ± 1.79 |
| 15 | *A. acutiloba* | 01-09-26 | Hokkaido, Japan | 6 ± 0.09 | 93 ± 1.12 |
| 16 | *A. gigas* | 01-09-17 | Chuncheon, Korea | 12 ± 0.09 | 245 ± 2.94 |
| 17 | *A. gigas* | 01-09-18 | Sokcho, Korea | 20 ± 0.15 | 285 ± 3.42 |

[a] Values are means ± SD, $n = 10–15$. [b] Seventeen populations of *Angelica* roots were collected fresh and dried under vacuum as described under Materials and Methods. [c] From 10 to 15 individual samples from each population were analyzed. All samples were collected in September–October, and they were all 2-year-old plants.

amounts of *Z*-ligustilide and ferulic acid than others, the overall clinical efficacies of these different *Angelica* roots have not been determined. Nevertheless, the contents of *Z*-ligustilide and ferulic acid could serve as markers for quality control. Indeed, these two chemicals have been used routinely on the markets, and they are responsible for part of the biological activity of *Angelica* roots (*14*).

The quality of crude drugs is closely related to their chemical constituents and could be assessed by a chemical pattern recognition method. By using the results from HPLC analyses, different samples of *Angelica* roots were identified with hierarchical clustering analysis. Within the same HPLC analytic conditions, 43 constituents including ferulic acid and *Z*-ligustilide were eluted, and the chemical data were obtained; these 43 constituents were quantified on the basis of their peak areas by using the peak area of *Z*-ligustilide as a reference standard. A matrix of 17 × 43 was obtained, which gave the content differences of 43 constituents among the tested 17 populations, as listed in **Table 1**. In the hierarchical clustering analysis, a method called average linkage between groups was applied, and Pearson correlation was selected as measurement; the result is shown in **Figure 3**. The 17 tested populations of *Angelica* roots were divided into three main clusters: samples 1−7 as cluster one; samples 8−13 as cluster two; and samples 14−17 as cluster three. This clustering agrees very well with the results of the contents of ferulic acid and *Z*-ligustilide. Members of cluster one were roots collected from Minxian of Gansu province and contained higher amounts of ferulic acid and *Z*-ligustilide than the others. Members of cluster two were collected from different provinces of China except Gansu, which contained lesser amounts of ferulic acid and *Z*-ligustilide, whereas members of cluster three were collected from Japan and Korea and contained the least amounts of ferulic acid and *Z*-ligustilide. The hierarchical clustering analysis suggests that *A. sinensis* root cultivated in Minxian of Gansu is distinct and that could be the cause of its superior quality.

For chemical fingerprinting of *Angelica* roots, HPLC peaks at an absorbance of 284 nm that had >1.6% of the total peak area, after normalization, were selected. The relative retention times ($t'_R$) of all these selected peaks, marked A−K, were calculated by using the retention time of *Z*-ligustilide as a reference standard (**Table 2**), and the fingerprints of randomly chosen samples from the 17 tested populations of *Angelica* roots

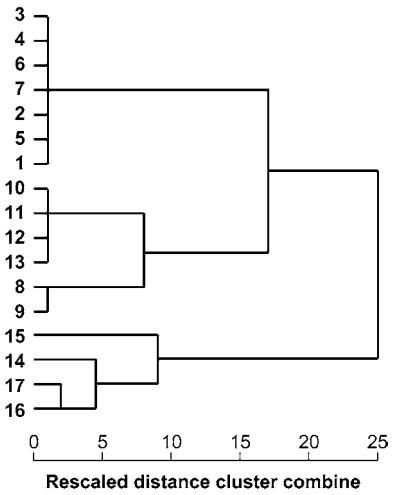


**Figure 3.** Hierarchical clustering analysis of different *Angelica* roots. The clustering was done by SPSS software from 43 HPLC peaks analyzed from the tested 17 *Angelica* roots; these samples were chosen randomly from the 17 populations, a single sample from each population, as listed in **Table 1**.

were acquired (**Figure 4**). Peaks corresponding to ferulic acid ($t'_R = 0.45$; marked D) and *Z*-ligustilide ($t'_R = 1.0$; marked J) were common in the 17 tested samples of three species of *Angelica* roots. The following peaks are characteristic and unique to the three *Angelica* species: $t'_R = 0.81$ for *A. sinensis*, $t'_R = 0.40$ for *A. acutiloba*, and $t'_R = 1.035$ for *A. gigas*. In addition, *A. acutiloba* and *A. gigas* are characterized by having extra peaks of $t'_R$ at 0.17, 0.25, 0.487, 0.654, and 0.671; all of these peaks are absent in *A. sinensis*.

In the Chinese Pharmacopoeia (*9*), two other *Angelica* species including *A. dahurica* and *A. pubescens* are listed for usage as traditional Chinese medicine, but their applications are very distinct from those of Radix Angelica. Moreover, the morphological properties of these two *Angelica* species are very different from those of *A. sinensis*; indeed, they all have different Chinese names. Therefore, roots of *A. dahurica* and A. *pubescens* have never been used as substitutes or counterfeits of Radix Angelica on the market. Besides the morphological distinction, roots of *A. dahurica* and *A. pubescens* contain coumarins, which can be easily distinguished from the roots of

**Table 2.** Relative Retention Times ($t'_R$) of Characteristic Peaks in the Chemical Fingerprints of *Angelica* Roots

| sample[a] | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | 0.455 | | | | 0.813 | 0.958 | 1.0 | |
| 2 | | | | 0.454 | | | | 0.812 | 0.958 | 1.0 | |
| 3 | | | | 0.454 | | | | 0.812 | 0.958 | 1.0 | |
| 4 | | | | 0.455 | | | | 0.812 | 0.958 | 1.0 | |
| 5 | | | | 0.455 | | | | 0.812 | 0.958 | 1.0 | |
| 6 | | | | 0.455 | | | | 0.812 | 0.958 | 1.0 | |
| 7 | | | | 0.455 | | | | 0.812 | 0.958 | 1.0 | |
| 8 | | | | 0.454 | | | | 0.813 | 0.958 | 1.0 | |
| 9 | | | | 0.453 | | | | 0.812 | 0.958 | 1.0 | |
| 10 | | | | 0.454 | | | | 0.813 | 0.958 | 1.0 | |
| 11 | | | | 0.455 | | | | 0.813 | 0.958 | 1.0 | |
| 12 | | | | 0.455 | | | | 0.813 | 0.958 | 1.0 | |
| 13 | | | | 0.454 | | | | 0.813 | 0.958 | 1.0 | |
| 14 | 0.174 | 0.253 | 0.404 | 0.458 | 0.487 | 0.654 | 0.671 | | 0.958 | 1.0 | |
| 15 | 0.173 | 0.248 | 0.403 | 0.458 | 0.487 | 0.654 | 0.671 | | 0.958 | 1.0 | |
| 16 | 0.168 | 0.258 | | 0.453 | 0.487 | 0.654 | 0.671 | | 0.958 | 1.0 | 1.035 |
| 17 | 0.166 | 0.258 | | 0.453 | 0.487 | 0.654 | 0.671 | | 0.958 | 1.0 | 1.035 |

[a] Seventeen populations of *Angelica* roots were collected fresh and treated as described under Materials and Methods. A single HPLC chromatogram at an absorbance of 284 nm was chosen randomly from the 17 populations, one single sample from each population, as listed in **Table 1**. The relative retention times ($t'_R$) of peaks designated A–K were calculated by using the retention time of *Z*-ligustilide as a reference standard of 1.0. D, ferulic acid; J, *Z*-ligustilide.

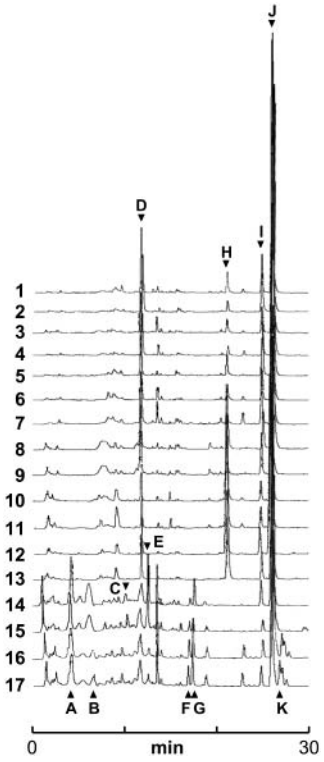


**Figure 4.** Chemical fingerprinting of *Angelica* roots. HPLC fingerprints at an absorbance of 284 nm from the 17 tested samples including the 3 different species of *Angelica* roots are superimposed. These samples were chosen randomly from the 17 populations, a single sample from each population, as listed in **Table 1**. The relative retention times ($t'_R$) of peaks A–K are shown in **Table 2**. D, ferulic acid; J, *Z*-ligustilide.

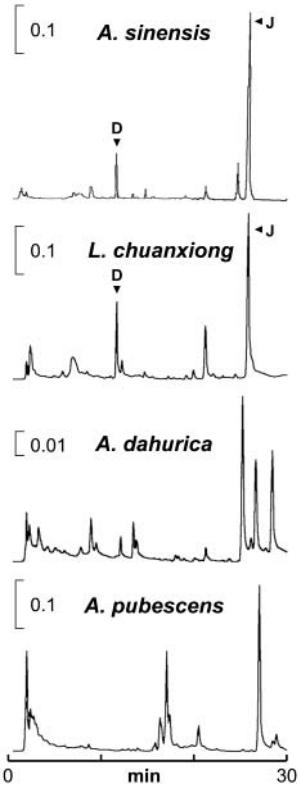


**Figure 5.** Chemical fingerprints of roots from *A. sinensis*, *A. dahurica*, *A. pubescens*, and *L. chuanxiong.* HPLC fingerprints from the four tested species as in **Figure 2** are shown. A typical profile is chosen from five tests of each population. Scale bars indicate the absorbance at 284 nm. The relative retention times ($t'_R$) of peaks D (ferulic acid) and J (*Z*-ligustilide) are shown.

*A. sinensis*. Here, the HPLC profiles of extracts derived from roots of *A. dahurica* and *A. pubescens* were also compared to that of *A. sinensis*, which showed distinct profiles (**Figure 5**). These results were in line with previous establishment by TLC as well as HPLC when using different parameters (*24*). Moreover, the root of *L. chuanxiong*, a member of Umbellaceae and commonly used as herbal medicine in China, is also known to contain ferulic acid and *Z*-ligustilide as its main constituents. However, the HPLC profile of *L. chuanxiong* roots as revealed here showed a distinct fingerprint from roots of *A. sinensis* (**Figure 5**).

In China, *Angelica* roots are processed usually by smoking to dry and/or by sulfur-smoking for anti-moth treatment; these treatments provide a longer preservation time (*10*). To study the chemical changes of *Angelica* roots during these treatments, we determined the contents of ferulic acid and *Z*-ligustilide in the smoking- and sulfur-smoking-treated roots, which were compared to the vacuum-drying method that was being used

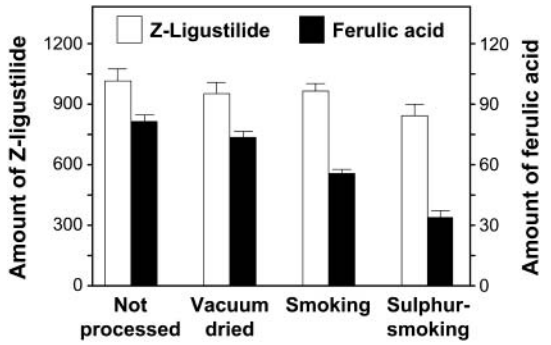


**Figure 6.** Reduction of ferulic acid after the preservation of *Angelica* roots. The amounts of ferulic acid and *Z*-ligustilide from *A. sinensis* roots of Gansu were determined as in **Figure 2**. The unprocessed roots served as a control. Vacuum-drying of roots was done by placing the fresh roots to dry for 2 days under the freeze-dryer. Smoking of roots was done by placing fresh roots under smoke generated by burning wet firewoods at ~40 °C for 2 days. Sulfur-smoking of roots was done similarly to the smoking of roots except with the addition of sulfur powder in the firewoods during the process. Smoking and sulfur-smoking are the two preservation methods commonly used in Gansu, China. The amounts of ferulic acid and *Z*-ligustilide are in mg/100 g of original root weight before any process had been done. Values are means ± SD, $n = 8$.

here. The results indicated that the content of ferulic acid was reduced by 30−60% after smoke processing, whereas the content of *Z*-ligustilide remained relatively unchanged (**Figure 6**). Therefore, we recommend that the traditional ways to process *Angelica* roots such as smoking and sulfur-smoking could result in the reduction of ferulic acid, probably due to the volatilization of ferulic acid. Processing techniques such as vacuum-drying should be considered, even though it is more costly.

The present studies provide genetic and chemical markers for authentic identification of common *Angelica* roots. Although the 5S-rRNA gene is highly conserved in higher eukaryotes, the spacer regions are variable in different species (*18*, *21*, *22*). As demonstrated here in *Angelica*, the diversity of the spacer regions can be used for genetic identification. Recently, DNA markers have been used extensively for the study of genetic diversity, phylogenetics, and taxonomy of plants, as well as for the identification of traditional Chinese medicines (*17*−*20*). DNA-profiling (DNA-based polymorphism assay) has several advantages over morphological and chemical analyses for the identification and discrimination of biological materials: (i) it analyzes the genotype rather than the phenotype and, therefore, is not affected by environmental factors; (ii) DNA can be prepared from small amounts of samples, and, using a PCR technology, nanogram quantities of DNA can be amplified to yield sufficient amounts of template DNA for molecular genetic analysis.

We successfully amplified the spacer domains of 5S-rRNA from different geographic strains of *A. sinensis*, and they showed no presence of intraspecies sequence variation. This perhaps could reveal differences of the quality of *Angelica* roots among different cultivated regions, which therefore should be a result of their ecological environment but not by diversity in their DNA sequences. Among different cultivated regions in China, Minxian of Gansu produces the best quality of *Angelica* roots, which could be due to its specific geographical location. Minxian is a located in the southwestern part of Gansu province, which has unique environmental properties that are very favorable for the cultivation of *Angelica* roots. Minxian is 2800−3700 m above sea level and receives >600 mL of annual rainfall, and the average temperature in a year is ~10−15 °C. Besides the geographical properties, farmers in Minxain have experience of cultivating *Angelica* roots for over 1000 years. Indeed, Minxian produces >90% of the total production of *Angelica* roots in China.

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