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Chromatographic and mass spectrometric fingerprinting analyses of *Angelica sinensis* (Oliv.) Diels-derived dietary supplements

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Abstract

Angelica sinensis (Oliv.) Diels (“Danggui” in Chinese) is one of the most commonly used Traditional Chinese Medicines (TCMs). It has been used to invigorate blood circulation for the treatment of anemia, hypertension, chronic bronchitis, asthma, rheumatism and cardiovascular diseases. There are a number of *A. sinensis*-derived dietary supplements in the U. S. markets. However, no study has been conducted to investigate the quality of these dietary supplements. In this paper, high-performance liquid chromatographic and flow-injection mass spectrometric fingerprints were both evaluated to assess the consistency of *A. sinensis*-derived dietary supplements. Similarity analysis was carried out on the high-performance liquid chromatographic (HPLC) fingerprints. Meanwhile, principal component analysis (PCA) was performed on the data obtained from flow-injection mass spectrometric (FIMS) fingerprints, which can analyze each sample in 2 min, compared to 30 min required for the chromatographic fingerprint. Principal component analysis (PCA) of the FIMS fingerprints was performed. Both methods show significant chemical differences between samples that may be due to differences in growing locations, growing conditions, harvesting times, and/or botanical processing. The loading plots obtained from PCA singled out the discriminatory ions that were responsible for chemical differences of *A. sinensis*-derived dietary supplements.

Keywords

Angelica sinensis (Oliv.) Diels; dietary supplements; Chromatographic Fingerprint; Flow-injection Mass Spectrometric Fingerprint; Principal Component Analysis

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Introduction

Angelica sinensis (Oliv.) Diels (“Danggui” in Chinese) is one of the most commonly used Traditional Chinese Medicines (TCMs). Its medicinal history can be traced back 2000 years in the famous *Shen-nung Pents’ao-ching*, a Chinese medical book. It has been used to invigorate blood circulation for the treatment of anemia, hypertension, chronic bronchitis, asthma, rheumatism and cardiovascular diseases [1–3]. Around 70 formulas containing *A. sinensis* are recorded in the Chinese Pharmacopoeia (Version 2010). It is also used as a health food product for women’s care in Europe and in America. The demand for *A. sinensis* and *A. sinensis*-derived dietary supplements (ASDS) is increasing throughout the world. However, scientific and systematic evidence for the stability and effectiveness of ASDS is limited.

It is well known that the curative effects of TCMs are based on the synergic effect of its multiple constituents. But, the contents of the complex chemical constituents in TCMs are influenced by many factors, such as growing locations (climates, soil constituents), harvesting times, plant parts, and processing methods [4, 5]. The analysis of one or a few so-called “active ingredients” may not be sufficient to assess the quality of a TCM, not to mention pharmaceutical preparations or dietary supplements.

Chromatographic fingerprinting method was accepted by World Health Organization (WHO) as a powerful tool for identification, quality evaluation, and determination of batch-to-batch consistency of multi-components TCMs and TCM-derived preparations [5 6, 7].

Although some studies using high performance liquid chromatographic (HPLC) fingerprints of *A. sinensis* have been published [8, 9], no study on ASDS has been reported. The aim, therefore, of the present study, was to develop fingerprinting methods to assess the consistency of ASDS. Both HPLC with diode array detector (DAD) and flow-injection mass spectrometric (FIMS) fingerprinting method were investigated. In addition, the components responsible for the chemical differences were pinpointed by the loadings plot of principal component analysis (PCA). The generated data provided valuable insight into the application of HPLC/DAD and FIMS fingerprints for quality assessment of ASDS.

Experimental

Materials and chemicals

An authentic sample of *A. sinensis* was obtained from the American Herbal Pharmacopoeia (AHP) (Scotts Valley, CA, USA).

Twenty-three commercial ASDS samples including 15 capsule samples, 5 liquid samples, and 3 powder samples from different manufacturers were purchased..

Optima*-grade methanol, acetonitrile, and water were purchased from Fisher Scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA). Mass spectrometry-grade formic acid was purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Instrumentation and conditions

An LCQ classic ion-trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with an Agilent 1100 HPLC system (consisting of a quaternary pump with a vacuum degasser, a thermostatic column compartment, an auto-sampler, and a diode array detector, Agilent Technologies, Palo Alto, CA, USA) was used in the present study.

Chromatographic fingerprinting Study—The chromatographic separations were performed on a reversed-phase C18 column (Kinetex C18, 150 mm × 2.1 mm i.d., 2.6 μ m, Phenomenex, Torrance, CA) at a flow rate of 0.25 mL/min. The HPLC mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) with the following linear gradient program: 0–3 min, 5–45% B; 3–15 min, 45%–66% B; 15–23 min, 66–95% B; 23–25 min, 95% B. UV spectra were acquired from 190 to 400 nm. The column temperature was maintained at 40 °C. The injection volume was 5 μ L. The LCQ mass spectrometer (MS) was used in the data-dependent scan mode to assist the peak identification. The parameters for the MS were: sheath gas flow rate, 80 (arbitrary units); aux gas flow rate, 10 (arbitrary units); spray voltage, 4.5 kV; heated capillary temperature, 220 °C; capillary voltage, 18 V; tube lens offset, 30 V. The most intense ion of each scan was selected for fragmentation with collision energy at 35%.

FIMS fingerprinting study—No analytical column was used for this study. Samples were directly injected into the MS through a C18 reversed-phase guard column (Adsorbosphere All-Guard Cartridge C18, 4.6 × 7.5 mm i.d., 5 μ m, Alltech Associates, Inc., Deerfield, IL), which serves primarily as an in-line filter to minimize the potential contamination for the MS system. Mobile phases consist of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with isocratic elution at 50:50 (v/v) was used. The flow rate was 0.5 mL/min. The injection volume was 5 μ L. Electro-spray ionization (ESI) was performed in positive ion mode from *m/z* 100 to 1000 to obtain the FIMS fingerprints. Three repeat analyses of the 24 different samples using the LCQ MS provided 72 spectra. The parameters for the LCQ MS were the same as mentioned above except the MS used the full scan mode.

Sample preparation

For solid samples, 200.0 mg of each sample was accurately weighed. Then, each sample was extracted using 10.00 mL of methanol for 30 min at room temperature with sonication (Branson 1200, Pegasus Scientific, Burtonsville MD USA). For liquid samples, computed volumes of each sample to give the equivalent to 200.0 mg of solid *A. sinensis* sample (according to label claims) were accurately pipetted and brought up to 10 mL with methanol, which was then sonicated for 30 min at room temperature. The extracted samples were centrifuged at 5,000 *g* for 10 min (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA) and were filtered through 0.22 μ m PVDF syringe filters (VWR Scientific, Seattle, WA) before analyses.

Data analysis

Similarity analysis—Similarity analysis of chromatographic fingerprints was performed by SpecAlign (Chemistry Department, Oxford University, Physical and Theoretical Chemistry Laboratory, Version 2.4.1). First, each chromatogram at 270 nm was exported to *.csv files from Xcalibur (Version 2.1). All the exported *.csv files were combined into one sheet in Microsoft Excel. To get rid of the solvent interferences, the intensities of the signals of all the chromatograms from 0 to 5 min were deleted and just those from 5 min to 25 min were used. Then, all the chromatographic fingerprints were aligned by using the fast Fourier transform (FFT)/peak matching combined method to adjust the detected peaks of individual spectra to those in the average spectrum. The final data matrix consisted of 24 rows (samples) × 3001 columns (intensities), and the correlation coefficients of similarities between the samples were calculated.

FIMS and PCA—FIMS spectrum of each sample was summed from 0 to 2.0 min. The FIMS fingerprint of each sample was a vector (ion counts with respect to mass-to-charge ratio, *m/z*, for a range from 100 to 1000). The spectra were exported with unit mass

resolution to Excel (Microsoft, Inc., Bellevue, WA) for preprocessing, i.e. combining the 72 spectra, sorting the data by sample names, and filled void masses in the matrix with 0s (each spectrum contained different numbers of ions since not all masses appeared in each spectrum). Three repeat analyses of the 24 different samples provided 72 spectra. The final data matrix consisted of 72 rows (samples) $\times$ 901 columns (the intensities of ions within the m/z range from 100 to 1000). The data matrix was then imported into SOLO (Eigenvector Research, Inc. Wenatchee, WA, USA). Normalization and then mean centering were performed before PCA.

Results and discussion

Chromatographic conditions

No HPLC/DAD method has been reported for analysis of the chemical constituents of commercial ASDS. After investigation of various HPLC columns, elution profiles, and mobile phases, the optimal gradient condition and corresponding HPLC column was selected (see Experimental section) to provide the separations of all of the main peaks. It was observed that most peaks in the chromatograms possessed strong UV absorbance at 270 nm. Hence, characteristic chromatographic patterns were obtained by using 270 nm as the detection wavelength. A representative chromatogram obtained from AS-1 is shown in Fig. 1. The retention time (t_R), UV, and MS data of each peak are listed in Table 1. Based on these data and references, peak 1, peaks 3~15 were tentatively identified (Table 1).

Similarity analysis of chromatographic fingerprints

The similarities and dissimilarities of chromatographic fingerprints of the tested samples were investigated by calculating correlation coefficients of the chromatograms [10]. The correlation coefficients obtained from SpecAlign were between 0 and 1. The closer the values are to 1, the more similar the two chromatograms are.

AS-1, the authentic sample of *A. sinensis* from AHP, was designated as the reference material, and the chromatogram obtained from AS-1 was regarded as the reference chromatogram in the present study. The correlation coefficient of each chromatogram to the AS1 were calculated (Table 2). Among all the ASDS samples, only the similarity values of Cap-15 (0.76), Liq-1 (0.73), Pow-1 (0.80), and Pow-3 (0.79) compared to AS-1 were above 0.70, indicating that the chemical profiles of them were more similar to AS-1 than others. Seven (Cap-4, Cap-8, Cap-9, Cap-12, Liq-3, Liq-4, and Pow-2) of the 23 ASDS samples had similarity values lower than 0.5. It was disappointing to see the similarity values of 30% of the ASDS tested were below 0.5 and only 17% were above 0.7.

FIMS fingerprint analysis

PCA is a sophisticated technique widely used for reducing the dimensions of multivariate problems [11]. As a non-parametric method, it makes no assumption about the underlying statistical data distribution [12, 13] and is primarily concerned with the transformation of a large set of related variables into new, uncorrelated variables, which are called principal components (PCs). As all the constructed PCs are orthogonal, the object scores can be plotted against each other to present the distribution of the objects in the space. Moreover, the contribution of each variable to a particular PC can also be calculated, giving each variable a weighting value or loading for a PC. High positive or negative loading values for variables indicate strong contributions to that PC.

2D PCA score plot

An example of the FIMS fingerprint of AS-1 is shown in Fig. 2. Fig. 3 shows the PCA score plot for the first 2 principal components (PCs). Clearly, Cap-2, Pow-2 and Liq-5 are far

away from other samples. The other samples are mainly clustered in four groups as I, II, III, and IV.

Group I contained samples AS-1, Pow-1, Pow-3, and Cap-15. The result was similar to the similarity test as the values of the three samples to AS-1 were 0.80, 0.79, and 0.76, respectively. They were the only three ones of which the similarity values to AS-1 were above 0.75.

Group II contained Cap-1, Cap-3, Cap-4, Cap-5, Cap-6, Cap-7, Cap-13, and Cap-14. With the exception of sample Cap-4, all samples in this group had similarity values greater than 0.59, indicating samples in group II were similar to the authentic raw material.

Group III contained Cap-8, Cap-9, Cap-10, Cap-11, and Cap-12 as well as Liq-1 and Liq-2. This group was where the similarity results do not agree with the PCA of FIMS, because 3 samples (Cap-8, 0.27; Cap-9, 0.47; and Cap-12, 0.34) have low similarity values and yet are close to group I in the PCA score plot.

Group IV contained Liq-3 and Liq-4, which are clustered at upper left. The similarity test values for the 2 samples are 0.34 and 0.36, respectively.

It was obvious that the results from the PCA score plot generally agree with the results obtained from a different technology, the similarity test of the chromatographic fingerprints.

3D PCA score plot

A 3D PCA scores plot (PC1, 2, 3) is shown in Fig. 4.

Samples in Group I of the 2D score plot: the same samples (AS-1, Pow-1, Pow-3, and Cap-15) still clustered together in the 3D score plot, confirmed the 2D grouping. Although Cap-13 from Group II appeared in the group, it was due to the specific viewing angle. Samples in Group II of the 2D score plot: the samples in group II are still clustered together in the 3D score plot except for sample Cap-13. By using the 3D plot, further separation of the group was achieved. Samples in Group III of the 2D score plot: While samples Cap-8, Cap-9, Cap-10, Cap-11, and Cap-12 clustered together in the 3D score plot as in the 2D score plot, samples Liq 1 and Liq 2 are now separated from the group. Samples in Group IV of the 2D score plot: Samples Liq-3 and Liq-4 still clustered together in the 3D score plot. Sample Cap-2 is closer to the group but it is due to the viewing angle. In summary, the additional information provided by the 3D PCA score plot separated samples Cap 13, Liq-1, and Liq-2 from their respective 2D PCA score plot groups.

PCA loadings plot

PCA loading plots were generated to find out the characteristic ions which are responsible for the distribution of samples in the PCA score plots. Generally, the loading of a variable on a PC reflects both how much the variable contributes to the PCs [14].

The loading plots on PC1 and PC2, and PC3 are shown in Fig. 5. The ions contributing most to the PC1 scores are m/z 191, 232, 266, 337, 381, 398, 520, and 640. The ions contributing most to the PC2 scores are ions at m/z 337, 478, 496, 520, 640, 696, 191, 232, 484, and 571. The ions contributing most to the PC3 scores are m/z 175, 337, 520, 191, 355, 571, 696, and 732.

The ions at m/z 191 and 232 are mainly from peak 7 to peak 9 with retention times at 13.50, 13.83, and 14.26, respectively. The MS spectrum is shown in Fig 6. 173.11 $[M+H-H_2O]^+$, 163.12 $[M+H-CO]^+$, 155.16 $[M+H-2H_2O]^+$, and 145.15 $[M+H-H_2O-CO]^+$ were found in its

MS² spectrum. The three peaks were tentatively identified as Butylphthalide, *E*-Ligustilide and *Z*-Ligustilide, respectively (Table 1) [9, 15–17, 20, 21]. The same peaks also have ions at *m/z* 232, which has an acetonitrile adduct ion of 191.

The ions at *m/z* 191, 381, and 398 are from peak 11 to peak 15 with retention times at 19.47, 19.64, 19.96, 20.37, and 20.56, respectively. The representative MS spectrum is shown in Fig 7, which gives a distinct *m/z* at 191[M+H]⁺, 381.10 [2M+H]⁺, 403.15 [2M+Na]⁺ as well as 337.32 and 398.09. The five peaks were annotated as the diligustilide [18] and its isomers of (*Z*, *Z'*-6, 8', 7, 3'-diligustilide [15, 19], Angelicide [15], Levistolide A [9, 15, 22], *Z*-ligustilide dimer [15], *Z*, *Z'*-3, 3', 8, 8'-diligustilide [15], respectively).

The ion at *m/z* 520 eludes at retention time of 15.3 min and does not belong to any major peaks in Fig. 1. It has protonated molecular ion [M+H]⁺ at *m/z* 520.33, [M+Na]⁺ ion at *m/z* 542.29, and [2M+H]⁺ ion at *m/z* 1039.43 in its spectrum. The ion of *m/z* 478 eludes at retention time of 15.2 min and does not belong to any major peaks in Fig. 1. It has a protonated molecular ion [M+H]⁺ at *m/z* 478.18, [M+Na]⁺ ion at *m/z* 500.30, and [2M+H]⁺ ion at *m/z* 955.09 in its spectrum. The ion at *m/z* 496 eludes at retention time of 16.6 min and does not belong to any major peaks in Fig 1. It has the protonated molecular ion [M+H]⁺ at *m/z* 496.28, [M+Na]⁺ ion at *m/z* 518.25, as well as [2M+H]⁺ ion at *m/z* 991.50 of this compound were found. Since all three ions eluded close to each other, they contained [M+Na]⁺ ions and [2M+H]⁺ ions. They are probably related to each other.

The ions *m/z* 484, 571, and 640 are all very weak peaks in the chromatograms, however, they do contribute significantly to the FIMS fingerprints. Satisfactory identifications of these minor compounds were not successful despite our best efforts.

Conclusion

A. Sinensis is widely used in TCM. There are commercial ASDS available in the U. S. markets. Both chromatographic and FIMS fingerprints have been applied to evaluate the consistency of the ASDS from different manufacturers in the present work. The similarity results obtained from chromatographic fingerprints and the PCA results from FIMS were similar. Wide varieties in the chemical compositions of the ASDS have been observed. The differences found between samples may be due to differences in growing locations, growing years, harvesting times, storage times and conditions, and processing methods. The analysis time of the FIMS method for each sample is 2 min and it only takes a few hours for method development, but it requires the use of a mass spectrometer. The chromatographic fingerprint method only uses a DAD detector, but it takes 30 minutes to analyze a sample and the method development time is long (about two weeks). The combination of the two methods can be used as a practical tool for assessment of the commercial ASDS products.

Acknowledgments

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RT: 0.00 - 25.00

NL:
3.34E5
nm=269.5-
270.5 PDA
AS1-POS

Fig. 1.
Chromatographic fingerprint of AS-1 detected at 270 nm. (Common peaks of all the tested samples are marked with 1 ~ 17).

Fig. 2.
Typical flow-injection MS full scan spectra obtained from AS-1.

Fig. 3.
2D PCA Score plot of the tested samples (PC1/PC2).

Fig. 4.
3D PCA score plot of the tested sample (PC1/PC2/PC3).

Fig. 6.
The MS and MS² spectra of peak 9 at retention time 14.26 min.

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AS1-POS #655 RT: 20.27 AV: 1 NL: 1.77E5

F: +c ESI Full ms [100.00-1500.00]

Fig. 7.
The MS and MS² spectra of peak 14 at retention time 20.37.

Table 1

Retention time (t_R), $UV\lambda_{max}$, m/z of the “Common Peaks”

Peak No.	t_R (min)	$UV\lambda_{max}$ (nm)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	MS^2 fragmentation ions	Identification	Reference
1	7.40	324	195.05	217.09	–	Ferulic acid	15, 18, 19, 21, 22
2	7.79	218	518.23	–	–	Unknown	–
3	7.96	220, 276	224.98	247.38	–	Senkynolide I/Senkynolide H	9, 15, 19, 20, 21
4	8.12	218, 276	207.23	229.08	–	Senkynolide F	15, 20, 21
5	11.26	220	206.90	–	–	Z-6,7- Epoxyligustilide	15, 20, 21
6	12.33	222	192.99	–	136.99, 147.10, 175.00	Senkynolide A	20, 21
7	13.50	222, 328	191.11	–	145.15, 148.98, 155.16, 163.12, 173.11	Butylphthalide	9, 15, 16, 17, 20, 21
8	13.83	212, 282, 328	191.13	–	145.15, 149.05, 155.16, 163.12, 173.11	E-Ligustilide	9, 15, 16, 17, 20, 21
9	14.26	224, 260sh	191.06	–	145.14, 149.06, 155.15, 163.13, 173.09	Z-Ligustilide	9, 15, 16, 17, 20, 21
10	14.47	218, 314	189.15	–	171, 153, 83	Z-Butyridenephthalide	9, 15, 20, 21,
11	19.47	222	381.08	403.16	191.07	Z,Z'-6, 8', 7, 3'-Diligustilide	15, 19
12	19.64	224	380.96	403.09	191.07	Angelicide	15
13	19.96	222	380.90	403.05	191.10	Levistolide A	9, 15, 22
14	20.37	224	381.09	403.19	191.10	Z-Ligustilide dimer	15
15	20.56	224, 276	381.04	403.15	191.10	Z,Z'-3, 3', 8, 8'-Diligustilide	15
16	21.65	224	782.38	804.41	282.18, 339.11, 385.37	Unknown	–
17	22.43	224	782.41	804.44	282.18, 339.11, 385.37	Unknown	–

Table 2

The similarity values of all the tested samples to AS-1.

Similarity to AS1	
Cap-1	0.6471
Cap-2	0.5035
Cap-3	0.6811
Cap-4	0.4293
Cap-5	0.6844
Cap-6	0.5890
Cap-7	0.6777
Cap-8	0.2749
Cap-9	0.4689
Cap-10	0.6381
Cap-11	0.5362
Cap-12	0.3355
Cap-13	0.5975
Cap-14	0.6529
Cap-15	0.7621
Liq-1	0.7342
Liq-2	0.5802
Liq-3	0.3410
Liq-4	0.3579
Liq-5	0.5206
Pow-1	0.7992
Pow-2	0.3985
Pow-3	0.7880