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Research article

FINGERPRINTS COMPARISON OF DIFFERENT PARTS IN RADIX *ANGELICA SINENSIS* BY UPLC AND GC-MS

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ABSTRACT: Radix *Angelica sinensis* (Danggui in Chinese) is a traditional Chinese medicine, which has long been used for tonifying blood and treating female irregular menstruation and amenorrhoea. The head, body, tail, and whole body of root exerted blood-stopping, blood-enriching, blood-breaking, and blood- activating effects respectively. However, these medicinal effects have received inconsiderable attention. In the study, the ultra-performance liquid chromatography (UPLC) and gas chromatography–mass spectrometry (GC–MS) were simultaneously applied to establish fingerprints for the first time, the four medicinal parts of RAS were characterized and compared through simultaneous and coupled with chemometrics. The results of UPLC and GC–MS demonstrated the diverse compositions of the different medicinal parts of RAS. Among the four different parts, the tail and whole body of RAS had the highest contents of butylidenephthalide and ligustilide, respectively. Chemical fingerprinting and quantitative analysis were performed to identify the contents of the compounds for quality control and to explore the cause of difference in pharmacological activity among the four medicinal parts. This study may serve as a reference for further research on the pharmacological activities of RAS.

Key words: *Angelica sinensis*; Ultra performance liquid chromatography; Gas chromatography-mass spectrometry

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INTRODUCTION

Radix *Angelica sinensis* (RAS) is widely used in TCM as a healthy food or a medicine for treatment of various diseases including irregular menstruation (Li, P.L et al, 2015). With the popularity of TCM in western countries, RAS has been introduced and applied in Europe and America duo to Its pharmacological function including invigorating blood circulation, enriching blood, regulating menstruation (Zhang, H.Y et al, 2012). Each part has significant distinctive clinical effects duo to its pharmacological activities (Qu, S.Y et al, 2004)]. The present study suggested that the head, body, tail, and whole body of RAS possess blood-stopping, blood-enriching, blood-breaking, and blood-enriching/ activating bioactivities respectively (Kang, J. et al, 2004).

Lv et al. elaborated that amino acids and essential oils are differently distributed on the head and tail of RAS (Wedge, D.E., Klun, J.A. (2009). In addition, Wang et al. reported that the four parts of RAS contain different contents of microelements (Piao, X.L et al, 2007). Although few papers expounded relevant study, the reasons led to efficacy difference of the distinctive parts of RAS remain to be emphasized.

Currently, six main active constituents in Chinese Angelica were simultaneous quantitated by HPLC-DAD (Li, Y et al, 2013). Moreover, the ferulic acid and phthalides of RAS were determined by UPLC-Q-TOF/MS (Wei, W.L., Huang, L.F. (2015). In addition, the bioactive compounds of RAS were separated and screened by CE or HPLC-MS (Hu, F. et al, 2015). However, only a few studies explored the chemical analysis and pharmacological activities of different parts of RAS, and the few samples was also an important question. Thus, the chemical fingerprint and composition of four parts were identified and analyzed to elucidate their difference of pharmacological activities. In the present study, UPLC and GC–MS coupled with principal component analysis (PCA) were simultaneously used to characterize different parts of RAS. We collected numbers of RAS samples and focused on the qualitative and quantitative analyses of different medicinal parts. This study may serve as a reference for the rational use of different parts of RAS.

Experimental

Chemicals, reagents and materials

Acetonitrile, formic acid, methanol, and ethyl acetate were purchased from Fisher Scientific Co. (Ma, USA). Acetonitrile and methanol were HPLC grade, and formic acid and ethyl acetate were analytical grade. All aqueous solutions were prepared using ultrapure water produced by Milli-Q system (18.2 MΩ, Millipore, Ma., USA). The standard ferulic acid (batch: 110773-201012) was purchased from National Institutes for Food and Drug Control (Beijing, China). Standard reagents (>98% purity) senkyunolide A (batch: MUST-10102309), n-butylphthalide (batch: MUST-12020706), ligustilide (batch: MUST-11072416), and butylidenephthalide (batch: MUST-12071103) were purchased from Must Co., Ltd. (Chengdu, China). The samples of RAS were collected on November 21, 2012 from Minxian City (Gansu Province, China), the genuine source of this medicinal herb. The samples were cut into three parts as head, body, and tail. These botanical materials were identified by Prof. Huang Linfang, and the specimens were deposited in the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, China. The chemical structures of ferulic acid and phthalide are shown in Figure 1.

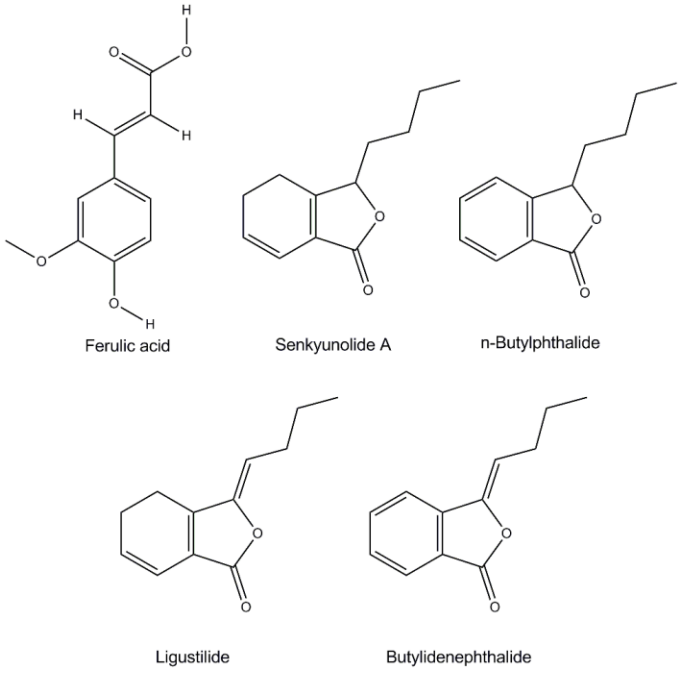

Fig-1: Chemical structures of ferulic acid and phthalides

Instrumentation and chromatographic conditions

Waters Acquity system (Waters, Milford, MA, USA) was equipped with a binary solvent delivery pump, an autosampler, and a PDA detector, and the system was connected to a Waters Empower 2 data station. Waters Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters, Milford, MA, USA) was used with column and sample temperatures of 35°C and 4 °C, respectively. The flow rate was 0.3 mL/min. The wavelengths were 261 and 281 nm. The mobile phase comprising acetonitrile (A) and 0.1% formic acid in water (B) was programmed as follows: 0–4 min: 95% (B); 4–7 min: 95%–76% (B); 7–8 min: 76%–72% (B); 8–10 min: 72%–50% (B); 10–12 min: 50%–30% (B); 12–14 min: 30%–0% (B); 14–15 min: 0% (B); and 15–16min: 0%–95% (B). An electronic analytical balance model AB135-2 (Mettler, Switzerland) and an electric-heated thermostatic water bath (Beijing Analytic Co., Beijing, China) were also used in this study.

An Agilent 6890 gas chromatograph (Agilent Co., Palo Alto, CA, USA) with a flame ionization detector (FID); an HP 5890-5971 gas chromatograph–mass spectrometer (HP, USA); and a Quartz capillary column gas chromatography column (HP, USA, 30 m × 0. 25 mm) were used. The column was initiated at 50 °C for 2 min. The temperature was increased at a rate of 10 °C/min to 250 °C for 10 min, and the split ratio was 1:10. The injection volume was 1.0 μL. The carrier gas was high-purity helium (99.99%). MS condition: electron ionization (EI) source; electron energy: 70 eV; ion source temperature: 230 °C; photomultiplier voltage: 1.25 kV; scan rate: 3.8 scan/s, from 30–550 amu with a solvent delay of 2 min. The GC was applied to investigate the optimal GC condition. Then, the mass spectra of the compounds were accepted when the matched quality was more than 70% compared with the NIST-Wiley Mass Spectra Library for identification (Muccio, A.D et al, 2006). Finally, PCA was performed by SIMCA-P 11.5 (Umetrics, Umea, Sweden) on the basis of the data from UPLC and GC–MS (Abdi, H., Williams, L.J. (2010).

Preparation of samples

For UPLC analysis, the five reference compounds were accurately weighed (1 mg), and dissolved in 10 mL volumetric flask with 70% methanol to produce standard stock solutions. The stock solution was diluted to yield a series of standard solution in the concentration range of 32–1722 μg/mL, 30–415 μg/mL, 156–722 μg/mL, 332–6786 μg/mL, and 33–2543 μg/mL for ferulic acid, senkyunolide, n-butylphthalide, ligustilide, and butylidenephthalide, respectively. Samples were ground into fine powder then passed through a 20 mesh (0.9 mm) sieve. Sample powder (0.2 g) was accurately weighed and transferred into a 60 mL round bottom flask. 70% methanol (20 mL) was added and refluxed for 30 min. When cool, the methanol was added to compensate for weight loss. After filtering through a 0.22 μm filter membrane, the filtrate was ready to be used.

For GC–MS analysis, the above standard solutions were prepared using ethyl acetate. In brief, 1 g of each sample was extracted twice with 100 mL of ethyl acetate through sonication, respectively. Then, the sample solutions were centrifuged (Sigma-1-14, Sigma Co., CA, USA), and the combined supernatants for each standard compound were filtered through 0.22 μm Millipore membranes before the analysis.

Methodology investigation

Methodology investigation of UPLC was used to evaluate the whole chromatographic system. Calibration curves of the standards were established with different concentrations (X) and peak areas (Y). The regression equations of ferulic acid, senkyunolide A, n-butylphthalide, ligustilide, and butylidenephthalide in UPLC were Y = 3E + 07X + 2835.3 (r = 0.9999), Y = 2E + 07X + 5256.2 (r = 1), Y = 7E + 07X + 295390 (r = 0.9991), Y = 6E + 07X + 11594 (r = 0.9999), and Y = 9E + 07X + 420771 (r = 0.9991), respectively, with corresponding linear ranges of 0.032–1.722, 0.030–0.415, 0.156–0.722, 0.332–6.786, and 0.033–2.543 mg/mL. Limit of detection (LOD) corresponds to the analyte amount for which the area is equal to three times of the chosen standard deviation, and limit of quantification (LOQ) corresponds to the analyte amount for which the area is equal to 10 times of the standard deviation chosen (Liu, C.X et al, 2007). Ferulic acid, senkyunolide A, n-butylphthalide, ligustilide, and butylidenephthalide had LODs of 4.15, 4.57, 5.33, 5.74, and 5.12 μg/mL, respectively, and LOQs of 11.06, 11.78, 12.56, 13.32, and 12.73 μg/mL, respectively. The inter-day and intra-day precisions, repeatability, and recovery rate were tested using RAS samples.

The inter-day precision was tested six times with RSD < 3.43%, whereas the intra-day precision was performed every 24 h for 3 d with RSD < 3.51%. The samples were detected at 0, 2, 4, 8, 12, 24, and 36 h with RSD of 0.76%–2.25%, which indicates the stability of the method. The recovery rates of the five standard compounds in the samples were 98.33%–102.22% with RSD of 1.16%–4.72%. These data indicated the validation of this chromatographic method.

The methodology adopted for GC was investigated using four standard compounds and samples through calibration curve, precision test, stability test, and recovery rate test. Different amounts (1, 2, 3, 4, and 5 µL) of each standard were analyzed, and the corresponding results were plotted to construct calibration curves. The regression equations for senkyunolide A, ligustilide, n-butylphthalide, and butylidenephthalide were y = 3434.3x + 62.73 (R2 = 0.9991), y = 14845x + 310 (R2 = 0.9992), y = 11476x + 160.87 (R2 = 0.9991), and y = 6245.8x + 120.38 (R2 = 0.9990), respectively. The precision was tested six times for each of the four standard compounds with RSD = 0.21%. The stability of the system was tested by detecting RAS samples at 0, 2, 4, 8, 12, 24, and 36 h with RSD of 0.76%–2.25%. The recovery rates of the samples were 98.33%–102.22% with RSD of 1.16%–4.72%.

RESULTS

UPLC analysis: The chemical fingerprint of RAS on the basis of UPLC analysis is presented in Figure 2A. The chromatographic conditions employed for UPLC were suitable for RAS. The maximum absorbances determined through UV spectrum scanning for ferulic acid, senkyunolide A, n-butylphthalide, ligustilide, and butylidenephthalide were observed at 321, 281, 261, 321, and 261 nm, respectively. The different parts of RAS showed obvious differences in chemical profiles. PCA was performed on the basis of the contents of ferulic acid, senkyunolide A, n-butylphthalide, ligustilide, and butylidenephthalide (Figure 2B). The results shown the separation of the different parts of RAS. The different chromatographic profiles characterized the head, body, tail, and whole body of RAS, which could be used to distinguish each part for quality control.

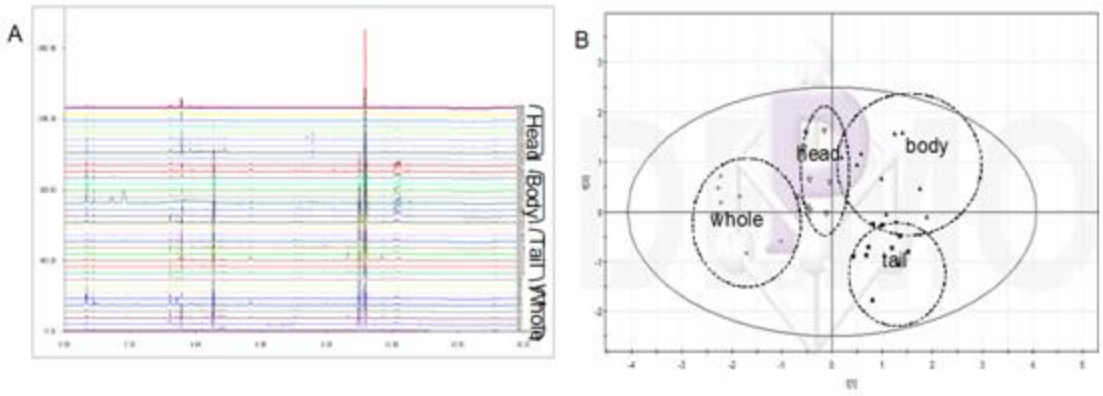

Fig-2: Fingerprint (A) and PCA (B) of different parts of *Angelica sinensis* by UPLC

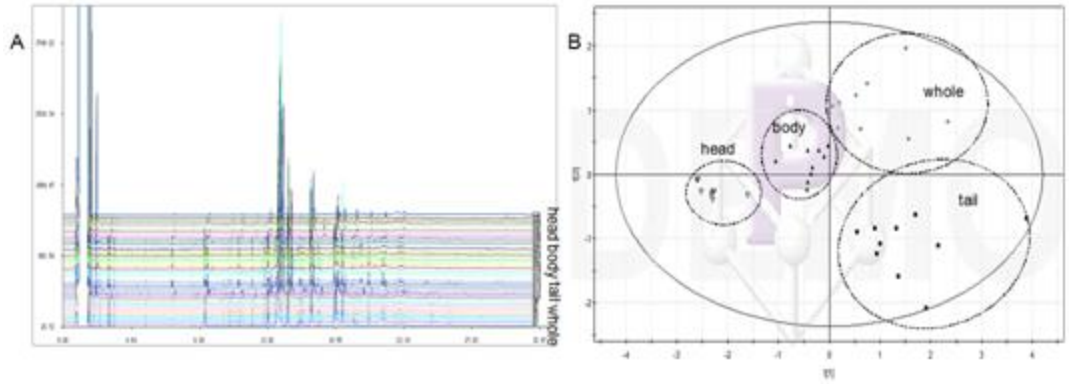

Fig-3: Fingerprint (A) and PCA (B) of different parts of *Angelica sinensis* by GC-MS

Table-1: The tentative compounds in Radix *Angelica sinensis* by GC-MS

NO.	Compounds	Tr	Mass weight	molecular formula	Area (%)
1	Ethylbenzene	12.321	106	C_8H_{10}	0.44
2	Benzene,1,3-dimethyl	12.473	106	C_8H_{10}	0.39
3	p-xylene	12.929	106	C_8H_{10}	0.18
4	Benzofuran-6-ol-one	13.898	310	$C_{18}H_{14}O_5$	0.36
5	Glycerin	14.216	92	$C_3H_8O_3$	0.70
6	1,3,6-Octatriene,3,7-dimethyl-,<E>-	15.212	136	$C_{10}H_{16}$	0.35
7	Tricosanoic acid	16.429	354	$C_{23}H_{46}O_2$	2.88
8	2,4,6-Octariene,2,6-dimethyl-	16.776	136	$C_{10}H_{16}$	0.03
9	Benzene,heptyl-	17.412	176	$C_{13}H_{20}$	0.01
10	1-Methylcyclohexa-1,3-diene	17.73	94	C_7H_{10}	0.02
11	Acetic acid	17.813	516	$C_{33}H_{56}O_4$	0.01
12	psi-carotene	17.965	600	$C_{42}H_{64}O_2$	0.01
13	14,15-Diepoxypregn-16-en-20-one	18.823	502	$C_{27}H_{34}O_9$	0.00
14	n-butylphthalide	18.934	190	$C_{12}H_{14}O_2$	3.89
15	1,3-Dioxane	19.363	538	$C_{35}H_{70}O_3$	0.01
16	3,4-didehydro-1,2,7,8-tetrahydro-1-methoxy -2-oxo-	19.625	582	$C_{41}H_{58}O_2$	0.01
17	Butylidenephthalide	19.778	188	$C_{12}H_{12}O_2$	4.20
18	1,1,2,2-tetrahydro-1,1-dimethoxy-	19.93	600	$C_{42}H_{64}O_2$	0.01
19	1,2-Benzenedicarboxylic acid	20.013	166	$C_8H_6O_4$	0.25
20	8-Benzoyloctanoic acid	20.414	248	$C_{15}H_{20}O_3$	0.02
21	1,4-cyclohexadiene-1,2-dicarboxylic anhydride	20.497	150	$C_8H_6O_3$	0.19
22	Senkyunolide A	21.147	192	$C_{12}H_{16}O_2$	2.06
23	cyclohexene	22.254	204	C_5H_{24}	0.04
24	cholestan-7-ol	23.126	508	$C_{34}H_{52}O_3$	1.61
25	4-ethylbenzoic acid	24.44	226	$C_{15}H_{14}O_2$	0.40
26	3-butylidene	24.716	188	$C_{12}H_{12}O_2$	1.58
27	Benzeneethanol	25.394	212	$C_{15}H_{16}O$	0.55
28	3-butylidene	25.464	188	$C_{12}H_{12}O_2$	0.14
29	Ligustilide	25.616	190.24	$C_{12}H_{14}O_2$	53.55
30	Methyl 3,5-tetradecadiy	28.245	234	$C_{15}H_{22}O_2$	0.07
31	dibutyl phthalate	28.535	278	$C_{16}H_{22}O_4$	23.66
32	2-Naphthalenone	30.541	180	$C_{11}H_{16}O_2$	0.61
33	5,7-Dodecadiyn-1,12-diol	34.069	194	$C_{12}H_{18}O_2$	1.67

GC–MS analysis

GC–MS is a powerful tool for identifying chemical components in essential oils (Wang, G.Y et al, 2003). Figure 3A presents the different chromatographic profiles of different parts of RAS. The N-dimensional vector approach was used to separate samples on the basis of the cumulative correlation of all data, wherein the vector that yielded the greatest separation among samples was identified without requiring prior knowledge of data sets. Mean-centered and par-scaled (scaled to square root of SD) mathematical methods were commonly used in PCA model (Ye, D.J., Zhang, S.C. (1999).

In Figure 3B, PCA presented the apparent separation in different parts of RAS on the basis of the contents of senkyunolide A, n-butylphthalide, ligustilide, and butylidenephthalide. A total of 33 compounds were identified by matching the retention time and mass weight of compounds detected in the samples with the database. The results are summarized in Table 1. These compounds were identified in all samples of RAS.

Table-2: The average content of ferulic acid and phthalides by UPLC (*n*=5)

Sample	Ferulic acid mg/g	Senkyunolide A mg/g	n-butylphthalie mg/g	Ligustilide mg/g	Butylidenephthalide mg/g
Head	0.694	0.128	0.144	2.025	0.940
Body	0.903	0.156	0.188	2.649	1.258
Tail	1.032	0.211	0.410	3.317	1.675
Whole body	1.027	0.111	0.267	3.128	1.455

Table-3: The average content of phthalides in *Angelica sinensis* by GC-MS (*n*=5)

Sample	n-butylphthalie mg/g	Butylidenephthalide mg/g	Senkyunolide A mg/g	Ligustilide mg/g
Head	0.0078	0.110	0.077	1.836
Body	0.0083	0.131	0.090	2.570
Tail	0.0077	0.252	0.040	2.717
Whole body	0.0140	0.132	0.118	2.965

DISCUSSION

Data analysis of UPLC

In this experiment, the contents of ferulic acid, senkyunolide A, n-butylphthalide, ligustilide, and butylidenephthalide were determined through UPLC. Phthalides such as n-butylphthalide, ligustilide, and butylidenephthalide are important compounds in volatile oils of *Angelica* species (Tao, J.Y et al, 1984, Ko, W.C. (1980). Methodology investigation showed the applicability of the methods adopted to determine all five standard compounds on the basis of linear regression, precision, repeatability, stability, and recovery rate test results. Four part of samples were analyzed, and the contents of the five compounds in different parts of RAS are listed in Table 2. The highest and lowest contents of ferulic acid, senkyunolide, n-butylphthalide, ligustilide, and butylidenephthalide were respectively identified in the tail (1.032, 0.211, 0.410, 3.317, and 2.275 mg/g, respectively) and head (0.694, 0.128, 0.144, 2.025, and 0.940 mg/g, respectively) of RAS. The contents of ferulic acid, n-butylphthalide, ligustilide, and butylidenephthalide were higher in the whole body (1.027, 0.267, 3.128, and 1.455 mg/g, respectively) than in the body of RAS (0.903, 0.188, 2.649, and 1.258 mg/g, respectively). As an exception, the content of senkyunolide A was lower in the whole body (0.111 mg/g) than in the body (0.156 mg/g) of RAS.

In TCM, the head, body, tail, and whole body of RAS had distinguished pharmacological effects. In specific, the head, body, and tail of RAS were utilized to promote hemostasis, enrich blood circulation, and eliminate extravasated blood, respectively. In modern pharmaceutical research, these five compounds were all important. As a common compound in medicinal plants, ferulic acid is known for its antioxidant, antimicrobial, anti-inflammatory, antithrombosis, and anticancer activities (Choi, Y.E., Park, E. (2015). Kang et al. [16] reported that an interaction exists between ferulic acid and human serum albumin. This result suggests that ferulic acid exerts effects on the human blood and the circulatory system. Senkyunolide A and ligustilide exhibit similar vasorelaxation effects on the cardiovascular system (Lv, G.H et al, 2004). Meanwhile, n-butylphthalide exerts oral antiplatelet and antithrombotic efficacy without perturbing systemic hemostasis in rats (Hyun, S.H et al, 2013). In addition, butylidenephthalide inhibits platelet aggregation by suppressing cyclo-oxygenase and interfering with calcium mobilization (Wu, L.B et al, 2013). Different parts of RAS demonstrated pharmacological effects on the circulation system, which could be combined in different concentrations and proportions of major compounds to further enhance the efficacy.

Data analysis of GC–MS

After performing chemometrics resolution on the GC–MS chromatographic profiles of all samples, the qualitative and quantitative results for each component can be determined. The presence and concentrations of four commonly detected bioactive components (senkyunolide A, n-butylphthalide, ligustilide, and butylidenephthalide) in different parts of RAS were compared through experiments. These compounds contribute to the pharmaceutical activities of RAS. These bioactive components were analyzed from RAS samples using the available corresponding standard compounds. On the basis of the results of GC–MS analysis, the different contents of the four main components among the different parts of RAS were compared under different measured distributions. The contents of main compounds in the different parts of RAS are shown in table. 3. As shown in table 3, the four parts had significantly different concentrations of the main components. The results further supported the differences observed on the proportion of the main compounds. Among the bioactive components, butylidenephthalide reportedly inhibits platelet aggregation and serotonin release, as well as increases coronary blood flow [22–24]. The results showed the rank of quality fraction of each main component as follows: n-butylphathlide, whole body (0.0014%) > body (0.00083%) > head (0.00078%) > tail (0.00077%); butylidenephthalide, tail (0.0252%) > whole body (0.0132%) > body (0.0131%) > head (0.011%); senkyunolide A, whole body (0.0118%) > body (0.009%) > head (0.0077%) > tail (0.004%); and ligustilide, whole body (0.297%) > tail (0.272%) > body (0.257%) > head (0.184%). The content and proportion of the main compounds significantly differed among the different parts of RAS, and these differences probably influenced the distinct pharmacological activities of each part. The variance in the pharmacological activities of the different medicinal parts of RAS should be considered for their proper use and efficacy.

CONCLUSIONS

This investigation focused on the samples of different parts of RAS, which exert distinguished pharmacological effects in traditional usages as blood-stopping, blood-enriching, blood-breaking, and blood-enriching/activating. Simultaneous analysis using UPLC and GC–MS coupled with PCA was performed for the first time to present the difference of sample profiles. Five main bioactive compounds of RAS were determined and separated through PCA. The results of comparative analysis showed that the distribution of components among the four parts was significantly different. The diverse pharmacological effects of the different parts of RAS can be ascribed to the various concentrations and proportions of the active compounds. Among the four medicinal parts, the tail had the highest content of butylidenephthalide. This finding could explain the blood-breaking activity of RAS tail. Meanwhile, ligustilide had significant content in the whole body of RAS and conferred blood-enriching/activating activity. A total of 33 volatile compounds were identified in the samples through GC–MS. This study may serve as a reference for further research on the different therapeutic effects of RAS.

Abbreviations

Ultra performance liquid chromatography (UPLC); Gas chromatography–mass spectrometry (GC-MS); Radix *Angelica sinensis* (RAS); Traditional Chinese medicine (TCM)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HLF conceived and designed the study. WWL performed the experiments and wrote the manuscript and analyzed data. WWL also searched and reviewed literature. All authors read and approved the final manuscript.

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