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Identification and quantification of sesquiterpenes and polyacetylenes in *Atractylodes lancea* from various geographical origins using GC-MS analysis

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Abstract: A convenient and sensitive GC-MS method was developed to identify thirteen sesquiterpenes and polyacetylenes (*e.g.* caryophyllene, γ-elemene, α-caryophyllene, β-selinene, isoledene, germacrene B, elixene, atractylone, hinesol, β-eudesmol, atrctylodin, atractylenolide II and acetylatractylodinol) in *Atractylodes lancea* (Thunb.) DC., Asteraceae. Among those compounds, four major components including atractylone, hinesol, β-eudesmol and atrctylodin were quantified with standards; contents of other components were estimated by using calibration curve of hinesol. In this study, we presented that the concentrations of those thirteen components varied drastically in *A. lancea* samples from different producing areas. Among those components, atractylenolide II and acetylatractylodinol were identified by GC-MS for the first time. A hierarchical clustering analysis based on relative peak areas of those thirteen components in total ion current (TIC) profiles was used to characterize *A. lancea* samples from different producing areas. Further clustering analysis showed that a simplified method with only four major bioactive components could be used to serve the same aim.

Article

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Introduction

The plant of *Atractylodes lancea* (Thunb.) DC., Asteraceae, named CangZhu in China belongs to the *Asteraceae* family. Its rhizome, commonly called *Rhizoma Atractylodis* is used as an important crude drug against rheumatic diseases, digestive disorders, night blindness and influenza in China (The State Pharmacopoeia Commission of People's Republic of China, 2010). This medicine has been well documented in Shen Nong Ben Cao Jing which is the first Chinese pharmacopoeia written in the Han dynasty about 200-100 BC (Editorial Committee of National Chinese Medical Manage Bureau Chinese Herb, 1999). The Korean and Japanese pharmacopoeias also have recorded this herb in traditional diuretic and gastric prescriptions.

The genus of *Atractylodes* consists of seven species, of which five species have been specifically found in China. The rhizomes of two species, *Atractylodes lancea* (Thunb.) DC. and *A. chinensis* (DC.) Koidz., named MaoCangZhu and BeiCangZhu in China separately, have been used as *Rhizoma Atractylodis* which embodied in the Pharmacopoeia of People's Republic of China 2010. The *Rhizoma Atractylodis* produced with *A. lancea* from Maoshan region of Jiangsu province have been considered to be the "famous region drugs" with highest-quality and has been evidenced by long-term clinical practice (Hu, 2001).

Nowadays, because of the scarcity of famous-region herbs from Maoshan, *Rhizoma Atractylodis* have been produced with *Atractylodes lancea* other areas. Thus, in order to determine clinical efficacy, the quality control of *Rhizoma Atractylodis* materials from different geographical origins is extremely important.

Previous studies have showed that *Rhizoma Atractylodis* are rich in essential oil, which mainly composed of sesquiterpenes and polyacetylenes compounds. It has been that volatile compounds are the major bioactive components of *Rhizoma Atractylodis*, and accounted for multi-activities of repelling convulsion, anti-inflammation, easing pain, sedation, anti-virus, anti anoxic and antihepatotoxic. Among those volatile components, some compounds including atractylone, hinesol, β-eudesmol and atrctylodin are considered as dominant bioactive ingredients (Ji et al., 2001). Atractylone, which accounted for 20% of the sesquiterpenoids, has been reported to prevent hepatotoxicity from CCl_4 in rats (Kiso et al., 1985). Furthermore, it has anti-microbial, anti-inflammatory and anti-ulcer activities (Guo et al., 2011; Liu et al., 2003). Hinesol could inhibit the secretion of gastric juice in rats (Nogami et al., 1986). It could enhance

the circulation and metabolism in the brain (Yamahara, 1991), and also inhibit the aggregation of platelets (Morita et al., 1992). β-eudesmol could significantly promote the gastrointestinal motility in mice (Wang et al., 2002). It has been demonstrated to be an inhibitor for tumor growth (Ma et al., 2008). It might also aid the development of drugs to treat angiogenic diseases (Tsuneki et al., 2005). Atractylodin could improve the delayed gastric emptying process (Nakai et al., 2003); also, it showed specific inhibitory effects on xanthine oxidase.

Rhizoma Atractylodis extract have been used as an important component in dozens of Chinese patent medicines. In addition, due to its various physiological functions, it has been widely considered as a potential herb medicine from which new drugs might be developed. However, the chemical compositions of *Atractylodes lancea* (the parent plant of *Rhizoma Atractylodis*) from different producing areas have drastic individual variations (Zhu et al., 1994; Hu et al., 2000). Therefore, it is very important to control the quality of *Atractylodes lancea* materials in the manufacture of related Chinese patent medicines. To our knowledge, so far only a few of those bioactive ingredients in *Atractylodes lancea* have been quantified by HPLC or GC methods due to the lack of reference standards (Takeda et al., 1995; Chen et al., 2007; Xie et al., 2008).

Gas chromatography-mass spectrometry (GC-MS) method could offer abundant information to identify volatile components in medicinal herbs. In present study, a convenient and sensitive GC-MS method has been developed for identification of thirteen components in *Rhizoma Atractylodis*. The contents of those thirteen components in nineteen *Rhizoma Atractylodis* samples from different geographical origin have been analyzed, and clustering analysis has been performed.

Materials and Methods

Plant material and chemicals

Nineteen samples(S1-S19) of *Rhizoma Atractylodis* were obtained from various geographical origins of China. Samples S1-S7 were "famous-region drugs" from Maoshan region, Jiangsu Province. S8 and S9 were from Nanshan and Yixing areas, Jiangsu Province, respectively. S10 and S11 samples were collected from Jimingshan and Yingshan region, Hubei Province. S12 was from Luotian area, Hubei Province. S13 and S14 were from Bozhou area, Anhui Province. S15-S17 were from Xinyang area, Henan Province. S18-S19 were from Shangluo area, Shaanxi Province. S1-S11 were collected in October 2008, and S12-S19 were collected in April 2009. All samples were identified to be the rhizomes of authentic *Atractylodes lancea* (Thunb.) DC., Asteraceae, by Professor J. G. Chao (School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China). Voucher specimens (voucher numbers of S1-S11: 2008OYCZ001-2008OYCZ011, voucher numbers of S12-S19: 2009OYCZ001-2009OYCZ008) are deposited in the Pharmacognosy Laboratory, School of Pharmaceutical, Jiangsu University. Samples were dried below 40 °C and then crushed into fine powder.

Atractylone, hinesol, β-eudesmol and atrctylodin, with >98% purity, were separated and purified in our laboratory. Their structures were confirmed by comparing their Mass Spectrometry and NMR data with those reported in references (Resch et al., 2001; Li et al., 2006; Zhao et al., 2006). Petroleum ether and ethyl acetate were purchased from Sinopharm Chemical Reagent (Shanghai, China).

Sample extraction

Powdered sample of *Rhizoma Atractylodis* (5.0 g) was extracted with 150 mL petroleum ether using ultrasonic extraction at room temperature for 30 min by three times. The three extracts of the same sample were pooled and concentrated under vacuum evaporation. A proper amount of completely concentrated extract (100 mg) was dissolved in 5.0 mL ethyl acetate and tested with GC-MS analysis.

GC-MS analysis

GC-MS was performed with an Agilent 6890 gas chromatography instrument coupled with an Agilent 5973 mass spectrometer. The data was analyzed with the Agilent ChemStation software (Agilent Technologies, Palo Alto, CA, USA). A capillary column HP-5 (30m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenylmethyl siloxane was used for separation. The column temperature was set at 85 °C and held for 5 min, then programmed at 3 °C min-1 to 185 °C and held for 10 min at the temperature of 185 °C, then at 5 °C min^{-1} to 250 °C, and finally, held for 5 min at the temperature of 250 °C. Split injection (2 μL) was conducted with a split ratio of 40:1 and high-purity Helium was used as carrier gas of 1.0 mL min^{-1} flow-rate. The spectrometers were operated in electron-impact (EI) mode, the scan range was 33-350 amu, and the ionization energy was 70 eV. The inlet and ionization source temperatures were 250 and 230 °C, respectively.

Hierarchical cluster analysis

The hierarchical clustering was performed with SPSS software (version 13.0, SPSS Inc., Chicago, USA). Average linkage between groups method was applied, and Squared Euclidean Distance was selected as measurement.

Result and Discussion

Identification of the bioactive components in Rhizoma Atractylodis

The total ion chromatograms of extracts from *Rhizoma Atractylodis* were shown in Figure 1. All the main components were separated completely, and thirteen of them were identified according to the mass spectra. By comparing with those of authentic standards from the NIST (National Institute of Standards and Technology), as well as by comparing with literatures (Chen et al., 2009; Guo et al., 2006; Yosioka et al., 1976) or standard compounds. Peaks 1-13 were identified as β-caryophyllene, γ-elemene, α-caryophyllene, β-selinene, isoledene, elixene, β-atirenene, atractylone, hinesol, β-eudesmol, atrctylodin, atractylenolide II and acetylatractylodinol, respectively. To our knowledge, this was the first report in which atractylenolide II and acetylatractylodinol were identified by GC-MS. Atractylenolide II and acetylatractylodinol were two bioactive components in *Atractylodes lancea*. Their physiological functions included anti-inflammation and delay of gastric emptying (Resch et al., 2001; Endo et al., 1979; Nakai et al., 2003).

Quantification of those thirteen components in Rhizoma Atractylodis

The selected ion monitoring (SIM) method was used for the quantification of thirteen components. The fragment ion *m/z* 108, which had the highest abundance, was used for the quantification of atractylone, similarly, *m/z* 161 was used for hinesol, *m/z* 149 was used for β-eudesmol and *m/z* 182 was used for atrctylodin. The MS fragmentation of hinesol was more similar to other identified components in *Rhizoma Atractylodis* than other three standards. Therefore, the relative contents of other components in *Rhizoma Atractylodis* were estimated by using calibration curve of hinesol.

The calibration curves obtained from the selected ions peak area of atractylone, hinesol, β-eudesmol and atrctylodin were linear over the range 0.006-1.018, 0.002-0.835, 0.001-0.540, 0.005-0.871 μg (absolute amount on column), with slope of 5×10^6, 3×10^6, 4×10^6 and 7×10^6, respectively. The coefficients of correlation (r) were 0.9995-0.9998. The limit of detection (LOD) and limit of quantitation (LOQ) were determined at a signal to noise ratio of 3:1 and 10:1. The LOD for atractylone, hinesol, β-eudesmol and atrctylodin were found to be 0.3, 1.0, 1.0 and 0.5 μg mL^{-1}, respectively. The LOQ for atractylone, hinesol, β-eudesmol and atrctylodin were 1.3, 4.0, 2.4 and 2.0 μg mL^{-1}, respectively.

The injection precision for atractylone, hinesol, β-eudesmol and atrctylodin was determined by injecting successively standard for six times. The relative standard deviations (RSD) of atractylone, hinesol, β-eudesmol and atrctylodin were 1.42, 1.04, 1.30 and 1.09%, respectively.

Six portions of the same sample (S14, from Bozhou area, Anhui Province) were extracted and analyzed using the above established method. The peak area of selected ions was stable. The RSD of repeatability for atractylone, hinesol, β-eudesmol and atrctylodin was 2.27, 1.01, 1.72 and 1.17%, respectively.

The stability of atractylone, hinesol, β-eudesmol and atrctylodin was also determined by inject freshly prepared standard solution for three times at 0, 2, 4, 8, 12, 16 and 24 h, respectively. The RSD of atractylone, hinesol, β-eudesmol and atrctylodin was 2.36, 1.60, 1.14 and 1.11%, respectively. Thus, the quantification of sesquiterpenes and polyacetylenes such as atractylone, hinesol, β-eudesmol and atrctylodin in *Rhizoma Atractylodis* could be preformed within 24 h after the sample extraction.

In order to validate the presented method, a known amount of atractylone, hinesol, β-eudesmol and atrctylodin was spiked into the sample (S14, from Bozhou area, Anhui Province) and extracted at specified conditions mentioned above. Six portions of the sample spiked with atractylone, hinesol, β-eudesmol and atrctylodin were extracted. The extract was injected to GC-MS, and the content of the analytes was calibrated. The recovery of the four tested components was between 98.4 and 101.1% with RSD of 1.02-1.86%.

The contents of thirteen identified components in *Rhizoma Atractylodis* were showed in Table 1. The data were used for quantitatively evaluating the quality of crude drugs, though some limits might exist. The results showed that the contents of thirteen identified components had significant individual variations in those *Atractylodis lancea* samples from different producing areas.

Comparison of the Rhizoma Atractylodis *samples from different productions areas*

At present, *Rhizoma Atractylodis* with various qualities from different producing areas were widely used in traditional Chinese medicine prescriptions, as well as the manufacture of some Chinese patent medicines. Our study clearly showed that the bioactive components in the samples of "famous-region drugs" were significant different from those in *Rhizoma Atractylodis* samples from other regions. Therefore, it was necessary to evaluate the quality of *Rhizoma Atractylodis* before it was used in clinical practice. In order to distinguish those Rhizoma Atractylodis materials from different producing areas, a GC-MS analysis of bioactive components was developed in this study. A hierarchical cluster analysis was performed based on relative peak areas of those thirteen components

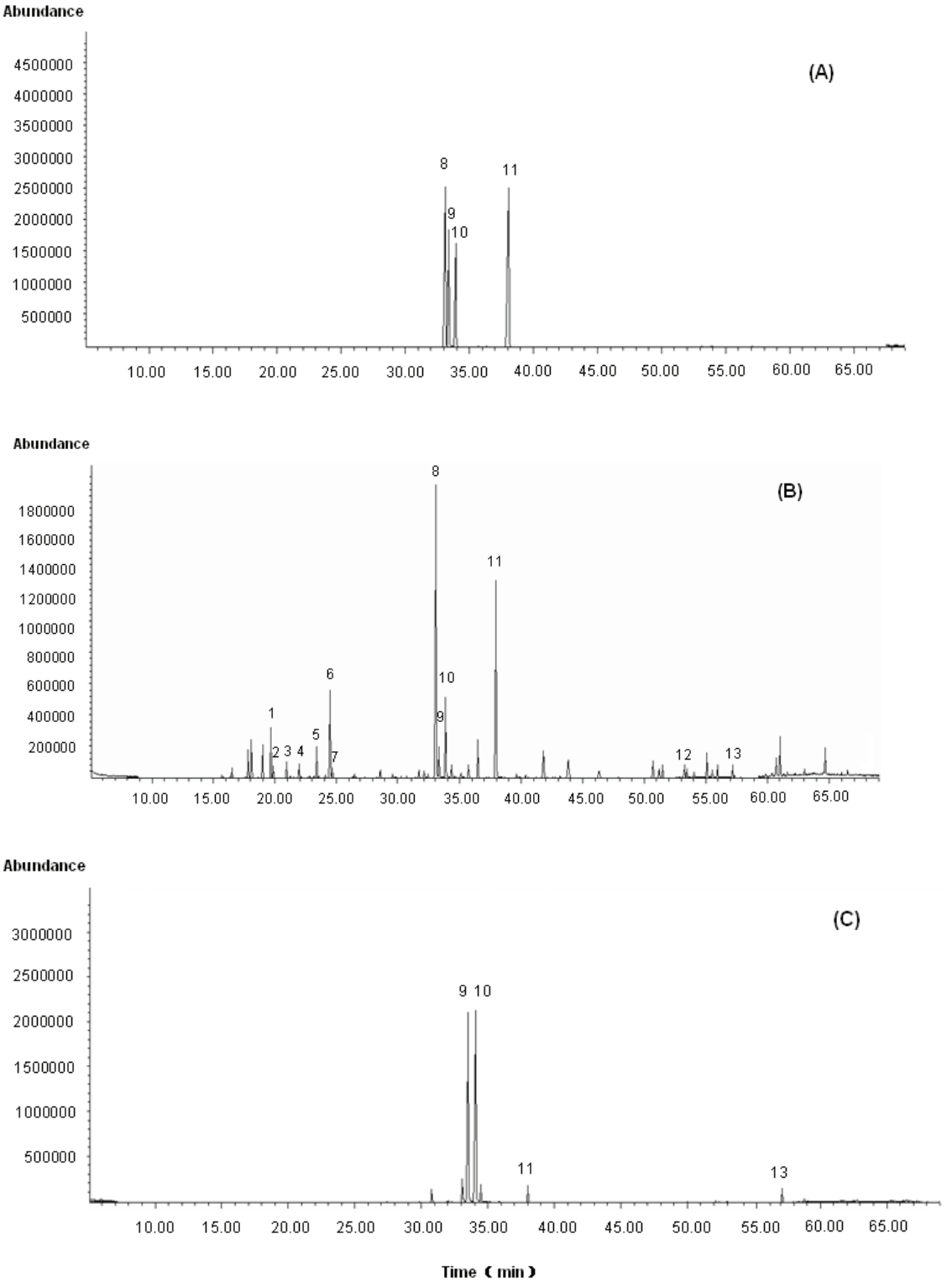

Figure 1. TIC of *Rhizoma Atractylodis* extracts from different producing areas in GC-MS analysis. A. Mixture of standards; B. Famous region sample collected from Maoshan region, Jiangsu Province; C. Representative sample collected from Xinyang area, Henan Province.

Table 1. Contents (mg/g) of thirteen compounds in *Rhizoma Atractylodis* samples from different producing areas.

Samples	β-Caryophyllene	γ-Elemene	α-Caryophyllene	β-Selinene	Isoledene	Elixene	β-Vatirenene	Atractylone	Hinesol	β-Eudesmol	Atrctylodin	Atractylenolide	Acetylatractylodinol	Total
S1	1.88(2.29)[a]	2.06(2.51)	3.14(3.82)	1.65(2.01)	3.23(3.93)	6.81(8.29)	1.90(2.31)	30.46(37.08)	3.03(3.69)	4.51(5.49)	18.68(22.74)	2.58(3.14)	2.21(2.69)	82.14
S2	2.36(3.12)	0.48(0.64)	2.42(3.20)	0.87(1.15)	3.08(4.08)	5.64(7.46)	1.15(1.52)	31.31(41.44)	2.58(3.41)	5.13(6.79)	16.28(21.54)	2.21(2.92)	2.05(2.71)	75.56
S3	4.13(4.72)	0.85(0.97)	4.08(4.66)	1.35(1.54)	6.89(7.87)	6.79(7.76)	1.52(1.74)	31.41(35.88)	2.78(3.18)	6.16(7.04)	16.92(19.33)	3.08(3.52)	1.57(1.79)	87.53
S4	3.44(4.15)	0.76(0.92)	3.19(3.85)	1.14(1.37)	4.81(5.80)	5.92(7.14)	1.49(1.80)	27.81(33.53)	4.72(5.69)	8.21(9.90)	17.81(21.47)	1.99(2.40)	1.66(2.00)	82.95
S5	3.12(3.62)	0.64(0.74)	2.80(3.25)	1.28(1.49)	6.21(7.21)	8.14(9.44)	1.42(1.65)	35.46(41.15)	4.28(4.97)	4.44(5.15)	15.12(17.54)	1.56(1.81)	1.71(1.98)	86.18
S6	2.44(3.19)	0.49(0.64)	2.16(2.82)	0.96(1.25)	5.59(7.31)	6.52(8.52)	1.31(1.71)	28.64(37.43)	4.42(5.78)	6.27(8.19)	14.27(18.65)	1.75(2.29)	1.69(2.21)	76.51
S7	5.26(5.56)	0.54(0.57)	4.85(5.12)	1.14(1.20)	6.08(6.42)	7.97(8.42)	1.34(1.42)	29.58(31.26)	5.60(5.92)	3.77(3.98)	23.43(24.76)	2.48(2.62)	2.59(2.74)	94.63
S8	5.99(6.72)	0.53(0.59)	5.71(6.40)	1.36(1.52)	8.34(9.35)	8.15(9.14)	1.65(1.85)	28.82(32.32)	0.40(0.45)	0.79(0.88)	25.64(28.75)	1.23(1.38)	0.56(0.63)	89.17
S9	—[b]	—	—	—	—	—	—	5.10(8.52)	18.04(30.12)	9.27(15.48)	10.59(17.68)	16.89(28.20)	—	59.89
S10	3.82(5.33)	—	3.35(4.68)	0.79(1.10)	5.75(8.03)	3.66(5.11)	0.72(1.00)	5.69(7.94)	20.76(28.98)	10.19(14.23)	13.91(19.42)	0.77(1.07)	2.22(3.10)	71.63
S11	0.66(0.89)	—	0.93(1.26)	1.04(1.41)	0.43(0.58)	8.68(11.75)	1.28(1.73)	25.02(33.86)	14.96(20.25)	8.66(11.72)	8.79(11.90)	1.75(2.37)	1.69(2.29)	73.89
S12	1.36(1.50)	—	1.32(1.45)	0.81(0.89)	3.89(4.28)	2.37(2.61)	0.75(0.83)	5.51(6.07)	42.42(46.70)	24.67(27.16)	5.51(6.07)	1.04(1.14)	1.19(1.31)	90.84
S13	1.31(1.91)	0.68(0.99)	1.57(2.29)	2.58(3.75)	2.97(4.32)	5.18(7.54)	0.96(1.40)	15.23(22.17)	4.51(6.56)	9.90(14.41)	19.06(27.75)	2.25(3.27)	2.49(3.62)	68.69
S14	0.86(1.26)	0.56(0.82)	0.91(1.33)	10.85(15.88)	0.99(1.45)	2.04(2.98)	0.66(0.96)	2.31(3.38)	6.85(10.02)	5.47(8.01)	32.02(46.86)	1.08(1.58)	3.73(5.46)	68.33
S15	—	—	—	—	—	—	—	—	42.5(55.20)	27.44(35.64)	3.16(4.10)	—	3.89(5.05)	76.99
S16	0.22(0.33)	—	1.00(1.52)	—	—	—	—	—	37.84(57.51)	19.83(30.14)	2.49(3.78)	—	4.42(6.72)	65.80
S17	—	—	—	—	—	—	—	—	26.99(61.06)	13.40(30.32)	1.40(3.17)	—	2.41(5.45)	44.20
S18	—	—	—	—	—	—	—	—	44.2(56.57)	29.11(37.26)	1.71(2.19)	—	3.11(3.98)	78.13
S19	—	—	—	—	—	—	—	—	45.74(60.00)	28.27(37.08)	0.86(1.13)	—	1.36(1.78)	76.23

[a]The data was presented as average of three replicates (R.S.D.<3%). The percentage of each component in total thirteen compositions was shown in parenthesis; [b] undetected.

in total ion current (TIC) profiles of those nineteen *Rhizoma Atractylodis* samples from different producing areas (Figure 2A) . Those nineteen samples were grouped into two main clusters. Sample from Nanshan region of Jiangsu province (S8), as well as those samples from Yingshan region of Hubei province and Bozhou area of Anhui province (S11, S13 and S14) showed the similar chemical compositions with those famous region drugs (S1-S7). That group of samples was characterized with having atractylone, hinesol, β-eudesmol and atrctylodin as their main components. Sample from Yixing areas of Jiangsu province (S9), as well as sample from Jimingshan and Luotian area of Hubei province (S10 and S12) showed the similar chemical compositions with those samples from Henan and Shaanxi province (S15-S19). That group was characterized with only containing hinesol and β-eudesmol as major components. In practical quality control procedures, to identify all those thirteen components in *Rhizoma Atractylodis* materials was over labor/time consuming. Thus, a simplified clustering method with four most crucial components (atractylone, hinesol, β-eudesmol and atrctylodin) was tested. The result of cluster analysis (described above) with those four compounds was similar with the previous result derived from 13 components (Figure 2B).

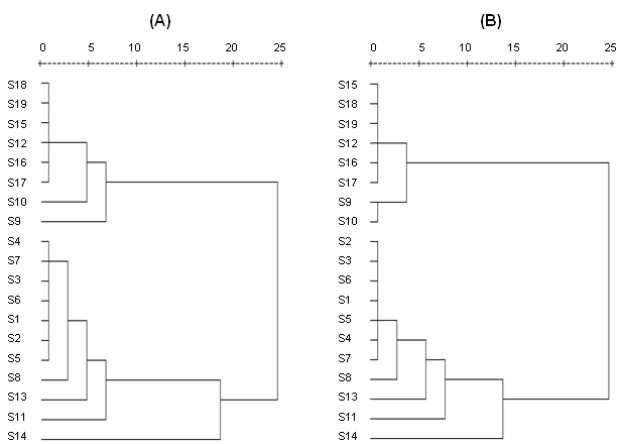

Figure 2. Dendrograms resulted from hierarchical cluster analysis. The hierarchical clustering was done with SPSS software. Average linkage between groups was used for clustering, and Squared Euclidean Distance was selected. A. Dendrogram resulted from the relative peak areas of those thirteen components in total ion current (TIC) profiles for the nineteen tested *Rhizoma Atractylodis* samples; B. Dendrogram resulted from four characteristics peaks (atractylone, hinesol, β-eudesmol and atrctylodin) in TIC profiles for the tested 19 *Rhizoma Atractylodis* samples.

Our study showed that geographical origin was one of the most important factors which confounded the compositions and contents of volatile components of *Atractylodes lancea* from different producing areas. Therefore, the overall pharmacological effects of those *Rhizoma Atractylodis* with different origins need to be further investigated. A quick and reliable determination of bioactive components in *Rhizoma Atractylodis* would be the foundation of further pharmacological evaluation. The GC-MS analysis followed with hierarchical clustering developed in our study could greatly facilitate the identification and quantification of bioactive components in Atractylodes lancea from different producing areas, as well as the research on their pharmacological effects.

In this study, for the first time, a convenient and sensitive GC-MS method was developed for identification and quantification of thirteen characteristic components in *Rhizoma Atractylodis*. A hierarchical clustering analysis was performed based on the contents of those thirteen (or four in simplified method) characteristic components to evaluate the qualities of *Atractylodes lancea* from different producing areas. The results showed that our method was a simple and effective assay in the quality control of *Atractylodes lancea* as a traditional Chinese medicinal herb.

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