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Discovery of characteristic chemical markers for inspecting sulfur-fumigated Radix Angelicae Sinensis by ultra-high performance liquid chromatography–quadrupole/time-of-flight mass spectrometry based metabolomics and chemical profiling approach

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ABSTRACT

An UHPLC–QTOF-MS/MS based metabolomics and chemical profiling approach was developed to identify the characteristic chemical markers for inspecting sulfur-fumigated Radix Angelicae Sinensis (S-RAS). Two sulfur-containing compounds in S-RAS were discovered and deduced to be (3*Z*)-6-sulfite-ligustilide and (3*E*)-6-sulfite-ligustilide. Furthermore, utilizing extraction ion method with the typical ion (*m/z* 271.06) of (3*Z*)-6-sulfite-ligustilide/(3*E*)-6-sulfite-ligustilide as the diagnostic ion, another six sulfur-containing components were determined in S-RAS, and identified as 6, 8-disulfite-ligustilide and 8-sulfite-ligustilide or their isomers. The generation mechanisms of these compounds were proposed as the addition reaction of sulfurous acid with (*Z*)-ligustilide and/or (*E*)-ligustilide, two major naturally occurring components in RAS, at the double bonds of C6═C7 and C3═C8 positions. Using (3*Z*)-6-sulfite-ligustilide and (3*E*)-6-sulfite-ligustilide as the chemical markers, fourteen of sixteen batches of commercial RAS samples were inspected to be S-RAS, suggesting that the newly generated sulfur-containing compounds could be used as characteristic chemical markers for inspecting S-RAS among commercial RAS samples.

1. Introduction

Radix Angelicae Sinensis (RAS), derived from the root of *Angelica sinensis* (Oliv.) Diels (Umbelliferae), is commonly used for treating a large variety of gynecological diseases such as dysmenorrhea, amenorrhea and to "enrich the blood" as an aid to recovery from blood loss after childbirth or surgery in Eastern Asia. Besides, it is also used as a health supplement for women's care in Europe and America by promoting blood circulation in treating menstrual disorders (Hook, 2014; Chinese Pharmacopoeia commission, 2000). In China, the primary geographic origin of RAS is Ming county of Gansu Province, where the low local average temperature (about 5.7 °C) is suitable for the storage of RAS. However, even at such optimal condition, the storage time of raw RAS is only six or seven months due to the moth and mold.

As one of the traditional process methods, sulfur-fumigation has been occasionally used for moth proofing and mold prevention of a few medicinal herbs (Chinese Pharmacopoeia commission, 2000). However, because of the lower cost and convenient in operation, sulfur-fumigation has been abused in the last decade during post-harvest handling and storage of many herbs (Liu, Liu, Li, Cai, & Cai, 2010). Although sulfur-fumigation has played a positive role in preservation of herbs, recent studies demonstrated that it could lead not only to the residue of hazardous substances such as sulfur dioxide and heavy metals (Liu et al., 2011), but also in chemical transformation of the original bioactive components in herbs (Wang et al., 2006). As a matter of fact, sulfur-fumigated herbs have been regarded as drugs of inferior quality by the Safe Food and Drug Administration of China since 2004 (Tellcent QQ. Available outline: http://finance.qq.com/a/20101015/002084.htm (in Chinese) (accessed on 12 Mar 2015)).

Sulfur-fumigation has also been frequently employed in processing fresh RAS. It was reported that sulfur-fumigation resulted in high residues of heavy metals and sulfur, and reduction of some beneficial trace elements in RAS (Lou et al., 2013), and global chemical differences were also demonstrated between sun-dried and sulfur-fumigated RAS using the Fourier transform infrared spectroscopy (FTIR) analysis (Lou et al., 2012). More recently, extensive study showed that sulfur-fumigation could significantly reduce the contents of bioactive chemicals and alleviate the pharmacological activities of RAS (Zhan et al., 2014). Therefore,

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developing a method to inspect the sulfur-fumigation induced degenerative RAS is crucial for the safe and effective usage of RAS.

Analytical methods using gas chromatography (GC) coupled with flame ionization detector (FID) (Yong & Patel, 2007; Yeh et al., 2012) or mass spectrometry (MS) (Yang, Chen, Lee, & Wang, 2008; Wedge et al., 2009) have been developed for analyzing RAS. However, considering the thermo-labile major components such as (*Z*)-ligustilide in RAS, GC might not be an ideal method for analyzing RAS. On the contrary, as a technique generally needs not too high temperature in operation, liquid chromatography, in particular, liquid chromatography–mass spectrometry (LC–MS) is characterized by its high sensitivity and resolution, which offers superior options to structurally elucidation of unknown compounds and enhances the opportunity to mine and uncover novel bioactive compounds of RAS (Wang, Liang, & Chen, 2007; Yang et al., 2006). Recently, the more powerful ultra-high performance liquid chromatography (UHPLC) coupled with quadrupole/time-of-flight mass spectrometry (QTOF–MS/MS) has been successfully used in metabolite profiling of medicinal herb (Xie et al., 2008). UHPLC–QTOF-MS/MS based metabolomics and chemical profiling approach, characterized by rapid processing of mass data and elucidating the identities of marker compounds, has been utilized to rapidly explore potential chemical markers to discriminate raw and processed herbs (Li et al., 2010).

In the current study, an UHPLC–QTOF-MS/MS based metabolomics and chemical profiling method was developed to rapidly identify the characteristic chemical markers for inspecting sulfur-fumigation induced degenerative RAS among commercial RAS samples.

2. Experimental

2.1. Chemicals and reagents

MS-grade methanol was purchased from Merck (Darmstadt, Germany). MS-grade formic acid was obtained from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water was prepared from a Milli-Q system (Millipore, Bedford, MA, USA).

Reference compounds of ferulic acid (**8**), senkyunolide I (**13**), senkyunolide H (**15**), senkyunolide A (**27**), (*Z*)-ligustilide (**31**), butylidenephalide (**32**), riligustilide (**35**) and levistolide A (**37**) were prepared by our research team with their identity confirmed by HR-MS, and ^{1}Hand^{13}CNMR analyses (Fig. S1) (Kobayashi, Fujita, & Mitsuhashi, 1984; Natio & Katsuhara, 1991). Their purities were determined to be higher than 95% by HPLC analysis.

2.2. Crude drugs

The non-sulfur-fumigated RAS (NS-RAS) samples prepared by the traditional sun drying (JSPACM-15-1, Batch No. 20121121) and sulfur-fumigated RAS (S-RAS) prepared by sulfur-fumigation of the fresh RAS samples (JSPACM-15-2, Batch No. 20121121) were collected from Min County of Gansu Province, the indigenous cultivation region of RAS. The other commercial RAS samples were obtained from different regions with detail information listed in Table 1. The voucher specimens of all these samples were deposited at the Department of Pharmaceutical Analysis and Metabolomics, Jiangsu Province Academy of Traditional Chinese Medicine. The herbal identities were authenticated by Prof. Song-Lin Li according to the monograph documented in China Pharmacopoeia (The State Pharmacopoeia Commission of P. R. China, 2010).

2.3. Liquid chromatography

UHPLC analysis was performed on a Waters ACQUITY UPLC™ system (Waters Corporation, Milford, MA, USA) equipped with a binary solvent delivery system and auto-sampler. The chromatography separation was achieved with a Waters ACQUTIY BEH C18 column (100 mm × 2.1 mm, 1.7 μm). The mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The UHPLC elution conditions were optimized as follows: linear gradient from 3 to 50% B (0–9 min), 50 to 60% B (9–11 min), isocratic 60% B (11–15 min), 60 to 70% B (15–16 min), isocratic 70% B (16–20 min), 70 to 97% B (20–21 min), isocratic 97% B (21–23 min), then back to 3% B in 2 min. The flow rate was set at 0.4 mL/min. The temperatures of column and auto-sampler were maintained at 35 °C and 10 °C, respectively. The injection volume was 2 μL.

Table 1
Origin of commercial RAS samples and inspecting results.

Code number	Origin	Batch number	Inspecting results
JSPACM-12-1	Nanjing City, Jiangsu Province	20130320	+
JSPACM-12-2	Nanjing City, Jiangsu Province	20130701	+
JSPACM-12-3	Nanjing City, Jiangsu Province	20130501	+
JSPACM-12-4	Nanjing City, Jiangsu Province	20130401	+
JSPACM-12-5	Guangzhou City, Guangdong Province	S20140701	−
JSPACM-12-6	Guangzhou City, Guangdong Province	S20140704	−
JSPACM-12-7	Zhuhai City, Guangdong Province	20130501	+
JSPACM-12-8	Zhuhai City, Guangdong Province	20121020	+
JSPACM-12-9	Zhuhai City, Guangdong Province	20110701	+
JSPACM-12-10	Jinzhong City, Shanxi Province	20120204	+
JSPACM-12-11	Jinzhong City, Shanxi Province	20130729	+
JSPACM-12-12	Jinzhong City, Shanxi Province	20120522	+
JSPACM-12-13	Jinzhong City, Shanxi Province	20130312	+
JSPACM-12-14	Shanghai	20120511	+
JSPACM-12-15	Shanghai	20130321	+
JSPACM-12-16	Suzhou City, Jiangsu Province	20130425	+

+: sulfur-fumigated; −: non sulfur-fumigated.

2.4. Mass spectrometry

Mass spectrometric analysis was performed on a SYNAPT G2-S QTOF-MS mass spectrometer (Waters MS Technologies, Manchester, UK), which was connected to the Waters ACQUITY UPLC system via an electrospray ionization (ESI) interface. Positive and negative ionization mode mass spectra were both collected on a MS^{E} mode with a scan range of 100–800 Da. During acquisition, alternating MS scans are collected at low (4 V) and high collision energy (20–50 V), providing precursor and fragment ions information, respectively. The mass spectrometer was operated with the following parameters: capillary voltage of 3 kV, cone voltage of 40 V, source temperature of 100 °C, desolvation temperature of 350 °C, desolvation gas flow of 500 L/h, and cone gas flow of 30 L/h. The Q-TOF acquisition rate was 0.2 s.

The mass accuracy and reproducibility were maintained using a LockSpray™. The $[M - H]^-$ (m/z 554.2615) and $[M + H]^+$ (m/z 556.2771) ions of leucine-enkephalin (1 ng/μL infused at 5 μL/min) were used as reference lock mass. Centroided data were acquired for each sample with dynamic range enhancement (DRETM) applied throughout the MS experiment to ensure accurate mass measurements. Data were collected and acquired using MassLynx V4.1 software (Waters Co., Milford, USA).

2.5. Sample preparation

2.5.1. Reference compound solutions

Stock solutions were separately prepared by dissolving the accurately weighed eight standard reference compounds (ferulic acid (**8**), senkyunolide I (**13**), senkyunolide H (**15**), senkyunolide A (**27**), (*Z*)-ligustilide (**31**), butylidenephthalide (**32**), riligustilide (**35**), and levistolide A (**37**)) with methanol. A mixed reference compound solution was obtained by mixing all the eight stock solutions above to obtain a final concentration of 3.70 μg/mL for ferulic acid, 4.40 μg/mL for senkyunolide I, 1.50 μg/mL for senkyunolide H, 11.00 μg/mL for senkyunolide A, 16.70 μg/mL for (*Z*)-ligustilide, 11.60 μg/mL for butylidenephthalide, 12.40 μg/mL for riligustilide