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Study of the destructive effect to inherent quality of *Angelicae dahuricae* radix (Baizhi) by sulfur-fumigated process using chromatographic fingerprinting analysis

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ABSTRACT

The after-harvesting sun-dried process of *Angelicae dahuricae* radix (Chinese name: Baizhi) was previously the traditional treatment for commodity. Over recent decades the natural drying process for some fleshy roots or rhizomes of Chinese materia medica has been replaced by sulfur-fumigation for curtailing the drying duration and pest control. We used high performance liquid chromatography (HPLC) and high performance thin-layer chromatography (HPTLC) fingerprinting analysis to investigate the potential damaging effect of the sulfur-fumigating process. The experimental conditions were as follows. HPTLC analysis was carried out on pre-coated silica-gel 60 plate, twice development was performed with two solvent systems (mobile phase) A, chloroform–ethyl acetate (10:1) and B, hexane–chloroform–ether (4:1:2); the fluorescent images were observed under UV 365 nm. HPLC was preceeded on Zorbax SB-C_{18} column; the linear gradient elution was conducted with mobile phase prepared from methanol–0.5% acetic acid; column temperature was at 25 °C; the detection wavelength was 250 nm. We found serious degradation of the majority of coumarins in sulfur-fumigated Baizhi. The destructive effect was manifested by the defaced chromatographic profile and verified by imitating the sulfur dioxide reaction with the constituents in Baizhi in the laboratory. It is suggested that sulfur-fumigation process is an unacceptable approach for processing herbal drugs.

1. Introduction

The traditional approach for treating herbal drugs after harvesting has been sun-drying or drying in shade. This would take a long time to dry for some of the fleshy roots or rhizomes of Chinese materia medica (CMM) [1,2]. Over recent decades an alternative method – fumigation by sulfur combustion in the closed cabinet – has replaced the natural dryness process for some commonly used CMM in some of the crude drugs processing workshops. It has really curtailed the dryness duration as well as the added role of pest control and bleaching the crude drugs or their slices for decoction (yin pian) to keep a better look. However, the sulfur dioxide generated during sulfur-fumigation period would inevitably react with ingredients in the herbal drugs in addition to the drying, desinsection and whitening effects. But the disadvantage of such processing method was covered up by the tangible advantage of such processing method. We investigated the destructive effect to the bioactive furocoumarins in *Angelicae dahuricae* radix (Baizhi) that was fumigated by combustion of sulfur using high performance thin-layer chromatographic (HPTLC) and high performance liquid chromatographic (HPLC) fingerprinting analysis.

2. Materials and instruments

2.1. The crude drugs

Twenty-one batches of sun-dried *A. dahuricae* radix (Baizhi) provided by Suining GAP cultivation base of Sichuan province (1 batch cultivated in 2005, 10 batches each in 2006 and 2007), and 17 batches of commercial samples purchased from market (2003–2007) were studied. All samples were identified by the author (X.H. Wang) and kept in our laboratory. The commercial samples were confirmed to have been treated by sulfur-fumigation using sulfite residue testing according to the state standard of sulfur dioxide residue test in food (GB/T 5009.34-2003).

2.2. Chemical reference substances

Imporatorin (purity >96%), isoimporatorin, and adenosine were purchased from the National Institute for the Control of

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Pharmaceutical and Biological Products, Beijing, China. Xanthotoxol, xanthotoxin, bergapten, oxypeucedanin (purities 92–96% for qualitative analysis) were purchased from Yousi Biotechnology Co. Ltd., Shanghai, China. Isopimpinellin (purity 95%) was provided by the State Administration of Traditional Chinese Medicine of China.

2.3. Chemicals

Methanol HPLC grade was purchased from Merck Chemical Corp., Darmstadt, Germany. Ethanol, chloroform, ethyl acetate, hexane, diethyl ether, all analytical grade, were purchased from Guangzhou Chemicals, Guangdong, China. Water for HPLC analysis was prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). HPTLC silica-gel 60 plate (10 cm × 10 cm, Lot: HX749869) was purchased from Merck.

2.4. Instrumentation

For HPTLC: LINOMAT 5 Semi-autosampler, Twin-trough Chamber for development and DigiStore documentation system (all from CAMAG, Muttenz, Switzerland). For HPLC: Agilent 1100 Series HPLC–DAD system with ChemStation (Agilent, Palo Alto, CA, USA), Sonication bath, 35 kHz and 360 W (Elma Co. Ltd., Pforzheim, Germany).

3. Experimental

3.1. Preparation of sample solution

Twenty milliliter of 70% ethanol was added to 1.0 g of powdered Baizhi sample in a Erlenmeyer flask. The mixture was sonicated twice for 30 and 15 min respectively, then filtered and evaporated to dryness at 90 °C on a water bath. The residue was dissolved in 70% ethanol and reconstituted to 10 ml as sample solution. Five milliliter of the solution was evaporated and adjusted to 2 ml for HPTLC analysis. Another 5 ml of the sample solution was filtered through a 0.45 μm filter membrane. The filtrate was for HPLC analysis.

3.2. Data processing method

The data acquired from HPLC fingerprints were processed by similarity assessment and Principle Component Analysis (PCA) carried out by using the Chromafinger software developed in our laboratory.

3.3. HPTLC study

3.3.1. Preparation of chemical reference substance (CRS) solutions

Appropriate quantities of CRS of xanthotoxol (0.1 mg/ml), xanthotoxin (0.1 mg/ml), oxypeucedanin (0.2 mg/ml), imporatorin (0.1 mg/ml), isoimporatorin (0.15 mg/ml), and isopimpinellin (0.3 mg/ml) were dissolved in methanol to produce the solutions as CRS solutions.

3.3.2. HPTLC conditions

Three microliter each of the sample solutions and 5 μl each of CRS solutions were applied as bands 8 mm in length onto the HPTLC silica gel plate; the interval between bands was 5 mm. The sample-loaded plate was dried in a vacuum desiccator over anhydrous P_2O_5 for 2 h prior to development. Ascending development was carried out twice at 20 °C in a twin-trough chamber containing the solvent system (mobile phase) in one trough was pre-equilibrated with solvent vapor for 15 min prior to development, the solvent system A of chloroform–ethyl acetate (10:1) for the first developing, running 45 mm; drying the plate with air stream; then the solvent system B of *n*-hexane–chloroform–ether (4:1:2) for the second, running 80 mm, and the developed plate was taken out of the chamber and the remnant solvent residue was removed with a hair-dryer. The fluorescent chromatogram was observed in UV cabinet under 365 nm and using the DigiStore documentation device captured the fluorescence image.

3.4. HPLC study

3.4.1. Preparation of solution of chemical reference substances (CRS)

Appropriate quantities of CRS of imporatorin, isoimporatorin, adenosine, isopimpinellin, xanthotoxol, xanthotoxin, bergapten and oxypeucedanin were dissolved in methanol to produce 0.1 mg/ml solutions. These were the CRS solutions for HPLC fingerprint identification.

3.4.2. HPLC conditions

For the HPLC fingerprint identification: column, Agilent Zorbax SB C_{18} column (5 μm, 4.6 mm × 250 mm) with a C_{18} guard column (5 μm, 4 mm L × 3.0 mm ID, Phenomenex, Torrance, U.S.A.); the mobile phase was consisted of 0.5% acetic acid in water (A) and methanol (B) using a linear gradient program of 0–100% (B) at 0–80 min with flow rate of 1.0 ml/min and column temperature at 25 °C; detection wavelength set at 250 nm.

4. The imitated reaction of sulfur dioxide with the constituents in Baizhi in laboratory

In order to confirm the impact of sulfur dioxide produced by combustion of sulfur to the main furocoumarins in Baizhi, a mimic reaction was carried out in the laboratory. Various amounts (0.1, 0.25, 0.5, 1.0 and 2.0 g) of sodium hydrogen sulfite ($NaHSO_3$) were added into 5 ml sample solution of sun-dried Baizhi sample for HPLC analysis (Section 3.3.2). Then 10% hydrochloric acid equivalent to the mol of $NaHSO_3$ was added and the mixture was heated at 80 °C on the water bath. The sulfur dioxide generated consequently reacted with the constituents in the sample solution until no ensuing reaction occurred, the solution was diluted with equal volume of 95% ethanol, mixed well, and filtered with Millipore 0.45 μm membrane, 20 μl of solution was taken for HPLC fingerprint analysis. The similar operation to reference substances of imporatorin, isoimporatorin, oxypeucedanin, bergapten, xanthotoxol and xanthotoxin were also carried out. The HPLC fingerprints of all the analytes were evaluated comparatively.

5. Results and discussion

5.1. Methodology optimization and validation

The sample solution preparation was compared by sonicated extraction (15, 30 min) and reflux extraction (single run: 60 min; twice run: 60 + 30 min) with ether, ethanol, methanol and 70% ethanol. Measuring the absorbance response of the major peaks in the chromatograms showed that the preference was given to 70% ethanol as solvent extracted by sonication (twice: 30 + 15 min) as described in Section 3.1.

Referring to the chromatographic condition optimization, HPTLC used HPTLC silica gel plate (Merck) and domestic HPTLC plate (Yantai Institute of Chemical Engineering Co. Ltd. Yantai, Shangdong, China), the comparative result showed that the resolution of HPTLC plate (Merck) was obviously better than that of Yantai plate. As for HPLC, upon testing of different columns, Zorbax SB-C18 (Agilent), Lichrocspher 100-RP-18 (Merck), and Hypersil BDS C18 (Elite, Dalian); mobile phases (methanol–water; methanol–0.5%

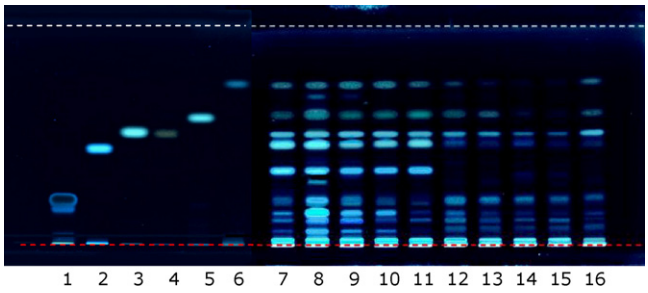

Fig. 1. HPTLC fluorescent images of coumarins and representive samples of Baizhi (1) xanthotoxol, (2) oxypeucedanin, (3) xanthotoxin, (4) isopimpinellin, (5) imporatorin, (6) isoimporatorin, (7–11) sun-dried samples and (12–16) sulfur-fumigated samples.

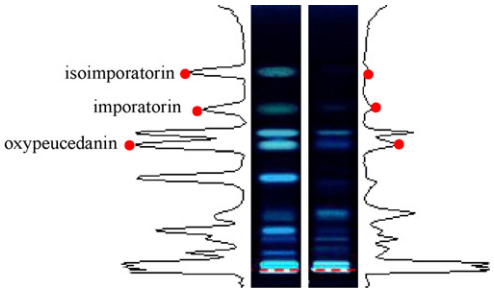

Fig. 2. Digital scanning profile of HPTLC fluorescent images showed almost all of the coumarins contained in natural dried Baizhi had been destroyed significantly in sulfur-fumigated sample. L: sun-dried Baizhi; R: sulfur-fumigated Baizhi.

acetic acid in water); column temperatures and detection wavelengths, the optimized incorporated conditions were set up as those mentioned in Section 3.4.2. The chromatograms had a wider tolerance to column temperature between 20 and 40 °C, and column temperature was maintained at 25 °C in this study. And the proposed method for HPLC fingerprint was validated in terms of precision, stability and repeatability. Precision was assessed with the solution of imporatorin, RSD of peak area was <2% (6×). Eight times injections of standard solution of imporatorin and sample solution within 48 h was used for evaluating stability, the entire chromatogram of each sample solution demonstrated high similarity (correlation coefficient: >0.99), and RSD of peak areas of imporatorin both in sample and standard solutions were <2%. The sample solutions were stable within 48 h. Six individual samples from same batch were extracted and processed according to the sample preparation procedures, and injected. The high similarity (correlative coefficient > 0.99) of the chromatograms confirmed the pretty good repeatability being acceptable for HPLC fingerprinting analysis.

5.2. The result of HPTLC fingerprint analysis

The HPTLC fluorescent images of Baizhi displayed more than 14 light blue or bluish-green fluorescent furocoumarins bands with weaker or stronger intensities to build a fingerprint of Baizhi. Observing the HPTLC image and the digital profile of Baizhi as a whole, the fluorescence bands of the furocoumarins of the sun-dried Baizhi were obviously stronger than that of sulfur-fumigated Baizhi (Figs. 1 and 2). The similarity and PCA differentiated clearly the two types of Baizhi (Fig. 3).

5.3. The result of HPLC fingerprint analysis

The HPLC fingerprint of Baizhi consisted mainly of 22 peaks, and the attribution of 10 peaks in the profile was confirmed by comparison of the retention time, UV spectrum of reference substances profile, spiking some of the chemical reference substances as well as referring to the data published in the literatures [3–5]. The attribution of the ten peaks was: adenosine (1), xanthotoxol (6), xanthotoxin (10), oxypeucedanin hydrate (12), isopimpinellin (14), bergapten (16), oxypeucedanin (18), imporatorin (19), cnidilin (21), and isoimporatorin (22). The peaks 10, 12, 16, 17, 18, 19, 21 and 22 dominated the profile of the sun-dried authenticated Baizhi; among them, peak 19 (imporatorin) was the uppermost (Figs. 4A and 5). The HPLC profile of sulfur-fumigated Baizhi demonstrated all the major furocoumarins were lost significantly, peak 19 (imporatorin) was lost by about 60%, peak 17 and peak 18 (oxypeucedanin) almost disappeared. However, the absorption abundance of peaks 3–11 (T_R = 18–44 min) increased in some extent (Figs. 4B and 5). Compared to the sun-dried Baizhi, the similarity of sulfur-fumigated samples was lower by 0.85 expressed by correlative coefficient (Fig. 6, L). The plot of PCA also revealed that the two types of Baizhi were neatly partitioned (Fig. 6, R). This observation denotes that sulfur-fumigation destroyed the constituents profile in varying degrees.

5.4. The impact of sulfur-fumigation to the dynamic changes of the main furocoumarins in Baizhi

As the testing results in Section 4, the HPLC fingerprint of the constituents in Baizhi, which was reacted with SO_2 showed oxypeucedanin (peak 18) was rapidly decomposed by only 0.1 g

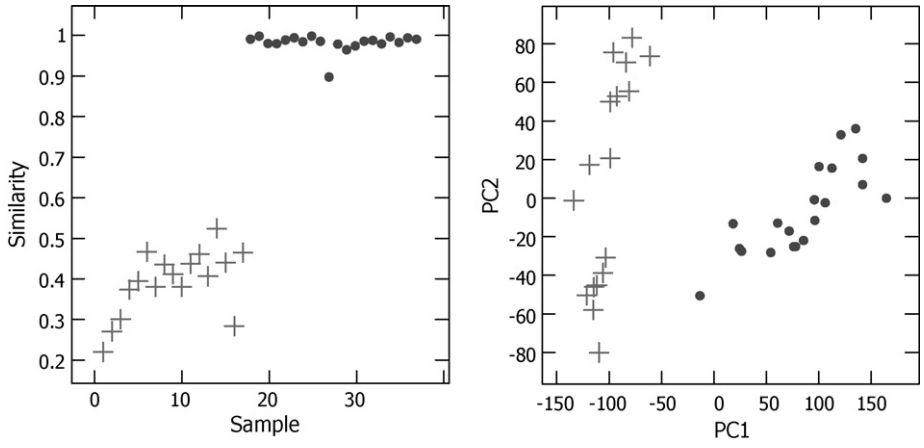

Fig. 3. Similarities (L) and principle component analysis (R) of the HPTLC fluorescent images of sun-dried Baizhi (+) and sulfur-fumigated Baizhi (●).

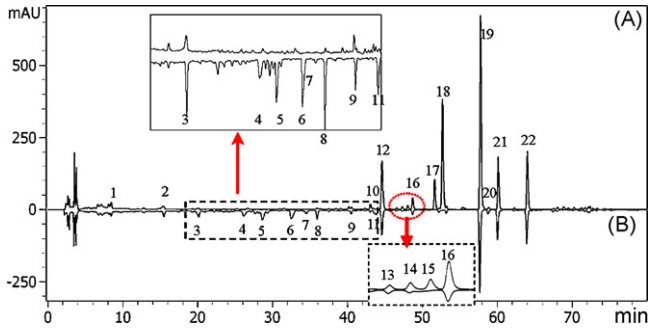

Fig. 4. Comparison of HPLC fingerprint common pattern of sun-dried (A) and sulfur-fumigated (B) samples peaks (1) adenosine; (6) xanthotoxol; (10) xanthotoxin; (12) oxypeucedanin hydrate; (14) isopimpinellin; (16) bergatpen; (18) oxypeucedanin; (19) imporatorin; (21) cnidilin; (22) isoimporatorin.

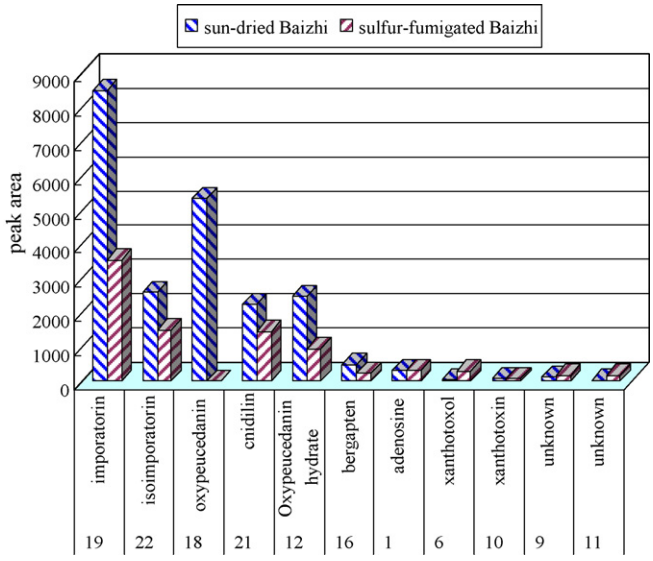

Fig. 5. Comparison of integrated peak areas between sun-dried Baizhi and sulfur-fumigated Baizhi.

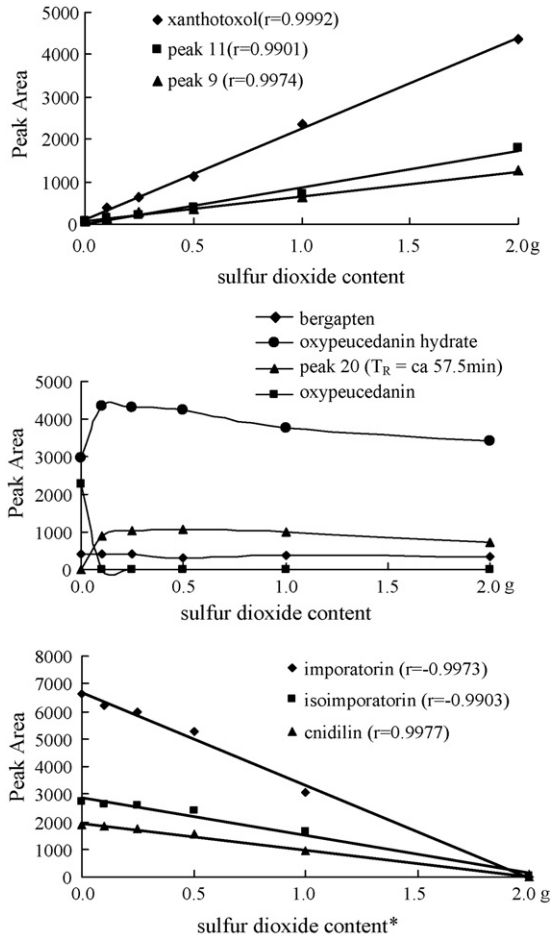

Fig. 7. The dynamic changes of some coumarins after reaction with SO_2. *Sulfur dioxide content was calculated as $NaHSO_3$ (g).

of $NaHSO_3$ and equal mol of HCl added. Imporatorin (peak 19), cnidilin (peak 21) and isoimporatorin (peak 22) were consecutively reduced in the wake of increased SO_2 amount with good linearity and positive correlation between peaks area and quantity of SO_2 ($r > 0.99$). However, xanthotoxol (peak 6), peak 9 and peak 11 were approximately double-increased. Bergapten (peak 16) and xanthotoxin (peak 10) were rather stable to SO_2. Peak areas of oxypeucedanin hydrate (peak 12) and an unknown peak located at 57.5 min increased when encountering with a little amount of SO_2 (≈0.1 g $NaHSO_3$), then reduced as the amount of SO_2 increasing (Fig. 7). A parallel study of reference substances showed bergapten (peak 16), xanthotoxol (peak 6), xanthotoxin (peak 10) are resistant to SO_2; imporatorin (peak 19) might convert into xanthotoxol (peak 6); isoimporatorin (peak 22) might probably convert into peak 11, according to the same converted principle of imporatorin, peak 11 was tentatively assigned as bergaptol; oxypeucedanin (peak 18) might be converted into oxypeucedanin hydrate (peak 12) and trace

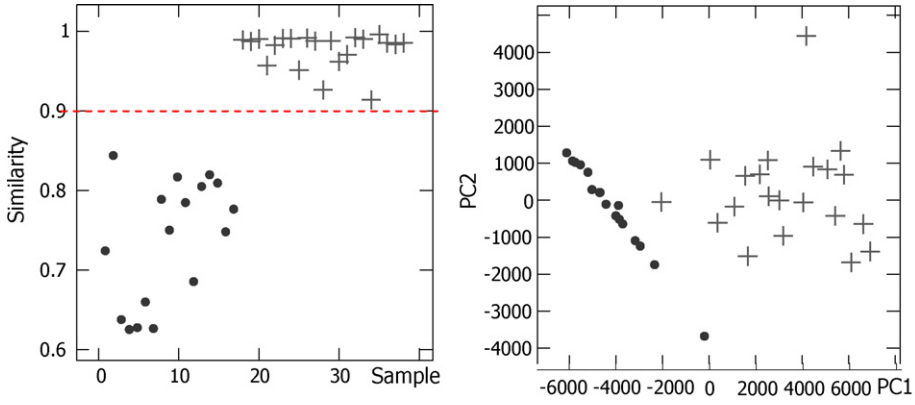

Fig. 6. Similarity of the HPLC fingerprints (L) and PCA project plot of HPLC fingerprints (R) of Baizhi. Sun-dried Baizhi (+); sulfur-fumigated Baizhi (●).

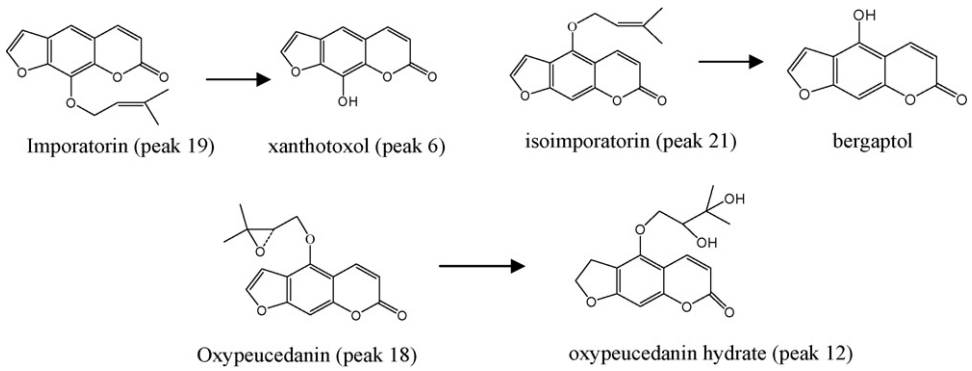

Fig. 8. Chemical structures of some coumarins in Baizhi.

xanthotoxol (peak 6) (Fig. 8) and other two unknown components with T_R at 52.4 min and 57.5 min.

All this above-mentioned changes also occurred upon imitating the method of sulfur combustion in an appropriate container in the laboratory, but the reaction progress was very slow due to insufficient sulfur dioxide generated within limited time and sulfur amount.

6. Conclusion

Using the method of sulfur combustion to drying some CMM with fleshy texture and rich starch or saccharide has for recent decades been a popular practice in some of the Chinese herbal drugs processing workshops for the sake of curtailing the drying process and bleaching the crude drug's darker outlook as well as moth proofing. However, simultaneously when the benefits are realized, the potential disadvantages occur. The chromatographic fingerprint analysis of Baizhi in this study revealed that during the crude drug subjects drying by the sulfur combustion over a rather long time, yet the simultaneous released sulfur dioxide reacts with the ingredients of *A. dahuricae* radix (Baizhi) causing serious loss of major bioactive furocoumarins such as imperatorin, isoimperatorin and oxypeucedanin. The HPTLC fluorescence images and HPLC profiles of Baizhi with and without sulfur-fumigation showed huge differences in its major ingredients, to estimate the integration peak areas of the profile of sulfur-fumigated Baizhi at the major furocoumarin, imporatorin lost approximately more than 60%, the most instable coumarin as oxypeucedanin even disappeared along with increasing of some minor furocoumarins which led the chromatographic fingerprint of Baizhi defaced drastically. This confirms sulfur-fumigation significantly damages the inherent quality of Baizhi. The destructive effect of sulfur-fumigation to the bioactive constituents has also been confirmed by imitating test in the laboratory. An unpublished figure in our laboratory showed that more than 40% of commercial crude drug of total 17 batches of Baizhi collected in the crude drug market contain less than 0.08% of imporatorin (content limitation in Chinese Pharmacopoeia 2005 edition) [1], contrary to the content of 0.16–0.31% of imporatorin in 21 batches of sun-dried Baizhi collected from Suining GAP Base of Baizhi in Sichuan province (Southwest China). Modern pharmacological and clinical studies revealed that the furocoumarins in roots of Baizhi possess various biological activities as anti-inflammation [6,7], anti-tumor [8], anti-microbial [9,10], central analgesic [11], heptoprotective [12], and the others. Significant loss of the furocoumarins implies a serious decline in its bio-activity. A published paper reported that the analgesic effect of sulfur-fumigated Baizhi decreased drastically than the sun-dried Baizhi [13]. The results of this study conclude that the sulfur-fumigation process bringing about significant loss of the main active constituents is consequently an unacceptable approach for processing herbal drugs.

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