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Original Research Article

Study on the Influence of Sulfur Fumigation on Chemical Constituents of *Angelicae dahuricae* Radix (Baizhi)

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Abstract

Purpose: *To study the influence of different sulfur fumigation time and dosage on the chemical constituents of Baizhi (Angelicae dahurica Radix).*
Methods: *The relationship of chemical constituents in Baizhi with different sulfur fumigation time and dosages was evaluated by high performance liquid chromatography (HPLC) fingerprint and chemometrics methods, including similarity analysis (SA), hierarchical clustering analysis (HCA) and principal component analysis (PCA). HPLC was carried out on a Diamonil C18 column; linear gradient elution was performed with acetonitrile and water; column temperature, injection volume of sample, flow rate, detection wavelength and testing time were 30 °C, 20 μL, 1 mL/min, 310 nm and 75 min respectively.*
Results: *The chemical constituents of Baizhi significantly ($p < 0.05$) decreased after sulfur fumigation though sulfur fumigation time and dosage were at low levels – 2 h and 25 g (sulphur)/10 kg (Baizhi), respectively.*
Conclusion: *Sulfur fumigation is not a desirable method for field processing of Baizhi even when sulfur fumigation time and dosage were short and small.*

Keywords: *Baizhi, Angelicae dahurica, Sulfur fumigation, HPLC-DAD, Fingerprint, Chemometrics methods*

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INTRODUCTION

Angelicae dahurica Radix, a common used Chinese medicine, has extensive pharmacological activities such as spasmolysis, analgesia, relieving asthma, etc. [1]. Currently, the problem about field processing of Baizhi has aroused wide attention. The medicinal part of Baizhi is fleshy root which is hard to timely dry and perishable [2]. Sulfur fumigation, typically used in the field processing of Baizhi all over China, can extend storage time and improve the appearance of traditional Chinese medicines (TCM) due to its preservative and bleaching effects. [3]. While a large number of studies have indicated that sulfur fumigation can not only cause the loss of biological active components but also create harmful residues of sulfur dioxide in TCM [3-8], and the negative impact of sulfur fumigation has aroused researchers' attention. In addition, it was found in our earlier study that active ingredients (coumarins) and pharmacological effects of Baizhi were evidently decreased after sulfur fumigation [9,10]. Previous studies mainly focus on the influence of the same sulfur fumigation time and dosages on Baizhi, and neglect the influence of different sulfur fumigation time and dosages on Baizhi. Moreover, there is a heated

debate about whether the chemical constituents of Baizhi were decreased by sulfur fumigation with short time and low dosages in the Good Agricultural Practices base (GAPB) of Baizhi, Sichuan Suining, China.

Our team designed a series of Baizhi samples, fumigated at varying sulfur fumigation time and sulfur doses, to study whether the chemical constituents of Baizhi were not damaged by sulfur fumigation with a short time and low dosages via HPLC fingerprint and chemo metrics methods such as similarity analysis (SA), hierarchical clustering analysis (HCA) and principal component analysis (PCA).

EXPERIMENTAL

Plant material

Baizhi samples were collected from Suining GAPB located in Sichuan (China) in September 2011, which were authenticated by Prof. Ma Yuying, a taxonomist in the College of Pharmacy, Chengdu University of TCM, Chengdu, China. A voucher specimen (63402/CDUTCM) was deposited in the herbarium of Chengdu University of TCM, Chengdu, China for future reference. The field processing information of all Bazhi samples are shown in Table 1.

Table 1: Processing methods and serial number

No.	Processing method	
	Sulfur fumigation time (h)	Sulfur dose (g/10 kg) (sulfur/medicinal material)
S1	24	25
S2	24	50
S3	24	100
S4	24	150
S5	24	200
S6	24	300
S7	24	500
S8	24	750
S9	2	150
S10	4	150
S11	6	150
S12	8	150
S13	10	150
S14	12	150
S15	18	150
S16	24	150
S17	36	150
S18	48	150
S19		lime-dried
S20		sun-dried

Standard compounds

Oxypeucedanin hydrate, byakangelicin, bergapten, isopimpinellin, neobyakangelicol, oxypeucedanin, byakangelicol, phellopterin, imperatorin and isoimperatorin were purchased from National Institutes for food and Drug Control or available in our laboratory.

Reagents

Methanol, tetrahydrofuran and acetonitrile were purchased from Fisher (HPLC grade, Fisher Scientific, Germany). Ultra-pure water was prepared by Milli-Q Advantage A10 (Millipore Crop., MA, USA). All other reagents were analytical grade.

HPLC fingerprint

Liquid chromatography

HPLC analysis was carried out on an Agilent Technologies 1200 system (Agilent Crop., MA, USA), equipped with a binary solvent delivery pump, an auto sampler and a DAD detector, and connected to an Agilent ChemStation. The chromatography was performed on a Diamonil C18 column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of acetonitrile (solvent A) and water (solvent B) with a linear gradient: 0 - 5 min (5 %, A), 5 - 15 min (5 - 27 %, A), 15 - 22 min (27 - 45 %, A), 22 - 52 min (45 - 58 %, A), 52 - 70 min (58 - 10 %, A). Column temperature, the injection volume of sample, the flow rate, the detection wavelength and the testing time were respectively 30 °C, 20 μL, 1 mL/min, 310 nm and 75 min.

Extracts of Baizhi

After processed Baizhi samples were ground into powder, twenty batches of samples were accurately weighed (about 0.5 g) and ultrasonic-extracted with 45 mL of methanol for 1 h at room temperature, and then the extract was filtered through a 0.22 μm membrane before analysis.

Preparation of mixing standard solution

Appropriate quantities of oxypeucedanin hydrate, byakangelicin, bergapten, isopimpinellin, neobyakangelicol, oxypeucedanin, byakangelicol, phellopterin, imperatorin and isoimperatorin were dissolved in methanol to produce mixed standard solutions, stored at 4 °C. The mixed standard solutions need to be standing to room temperature and then were filtered through a 0.22 μm membrane filter before analysis.

HPLC fingerprint method validation

The feasibility analysis of the HPLC fingerprint method of determining processed Baizhi samples was evaluated by precision, stability and reproducibility experiments which can be conducted though HPLC analysis mentioned above to determine chemical constituents in Baizhi samples at different times. Common peaks in HPLC fingerprint are defined as the chromatographic peaks which are appeared in all the samples. The relative standard deviation (RSD) values of retention time and peak area of common peaks with respect to the reference peak (imperatorin) were calculated to verify the precision, stability and reproducibility of the developed method.

Data analysis

Similarity analysis (SA), performed by Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A), was used to establish characteristic fingerprint and calculate similarity among samples. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) , performed by Statistical Product and Service Solution 19.0 (SPSS 19.0), were used to establish clusters and comprehensive evaluation of samples of various field processing methods. All peak data were represented as mean ± standard deviation (n = 5), which were analyzed by the two-tailed Student's t-test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Optimization of chromatographic conditions

The influence of mobile phases including methanol-water, methanol-tetrahydrofuran-water and acetonitrile- water, columns containing Diamonil (C18 4.6 mm × 250 mm, 5 μm) and Dikma (C18 4.6 mm × 250 mm, 5 μm), column temperatures including 25 °C and 30 °C on theoretical plates, resolution, reproducibility and tailing factor were studied, and the results indicated that acetonitrile-water, Diamonil C18 column (4.6 mm × 250 mm, 5 μm) and 30 °C column temperature could obtain desirable theoretical plates, resolution, reproducibility and tailing factor. In addition, the detection wavelength was identified as 310 nm by DAD three-dimensional map.

Optimization of sample preparation process

In this study, the influences of four types of extraction solvents (ethyl ether, ethyl acetate, 95 % ethanol and methanol) and three types of extraction methods (ultrasonic, reflux and cold soak) on extraction efficiency were investigated, and the results suggested that methanol and ultrasonic extraction was the best extraction solvent and method. In addition, the influences of other extracting conditions including extraction solvent volumes (30, 45 or 60 mL) and extraction time (30, 45, 60 or 75 min) on extraction efficiency also were explored. All the results suggested that ultrasonic with methanol (45 mL) for 75 min was the simplest and most efficient method for extraction of sample.

HPLC fingerprint method validation

The RSD of relative retention time and relative peak areas of common peaks were less than 1 and 5 percent separately, which were in conformity with the guideline of technology requirements about the research of TCM injection fingerprint (provisional, the state administration of TCM), and thus the developed HPLC fingerprint method was feasible in terms of precision, stability and reproducibility.

Identification of chromatographic peaks

Ten chromatographic peaks of Baizhi sample was identified by comparing retention time and UV absorption curve of samples with mixed standard solution. The results are presented in Figure 1 and Table 2.

Table 2: Retention time of standard substances

No.	Compounds	Retention time (min)
4	oxypeucedanin hydrate	22.811
5	Byakangelicin	23.292
7	Bergapten	27.438
8	Isopimpinellin	29.727
10	neobyakangelicol	33.006
11	Oxypeucedanin	33.612
13	Byakangelicol	35.661
14	Imperatorin	43.257
15	Phellopterin	46.577
17	Isoimperatorin	50.646

HPLC fingerprint analysis of Baizhi samples

Eighteen batches of Baizhi samples, processed with different sulfur fumigation time and dosages, were analyzed to study the characteristic

fingerprint according to the established HPLC-DAD analysis method, whose HPLC chromatograms were imported into Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A) to generate the characteristic fingerprint including nineteen characteristic peaks (1 - 19) within 75 min by multipoint correction and automatic matching. The results are shown in Figure 2.

Similarity analysis (SA)

The similarities of 18 batches of Baizhi samples were evaluated by the correlation coefficient (median) and time window (0.5) in Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004 A). The results are displayed in Table 3.

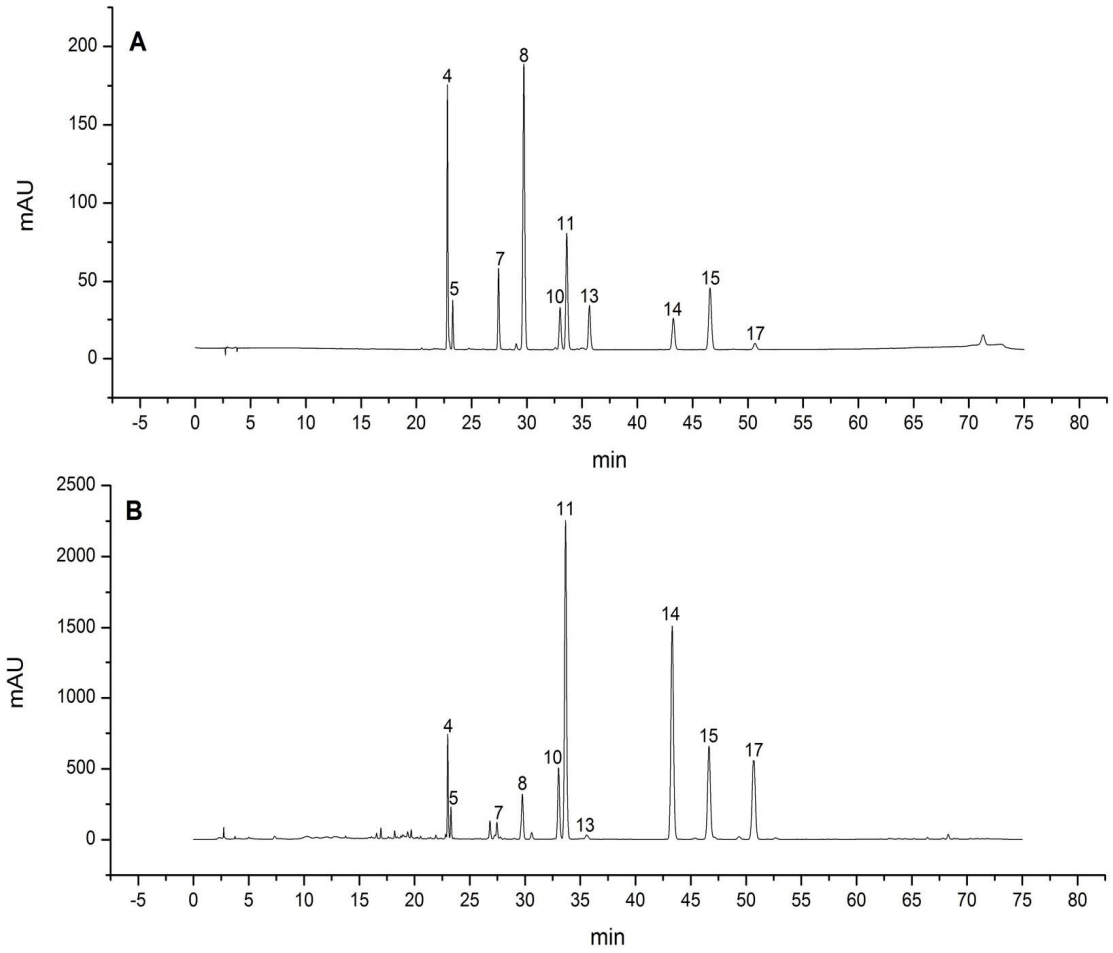

Figure 1: Chromatogram of coumarins standard substances (A) and Baizhi sample (B).

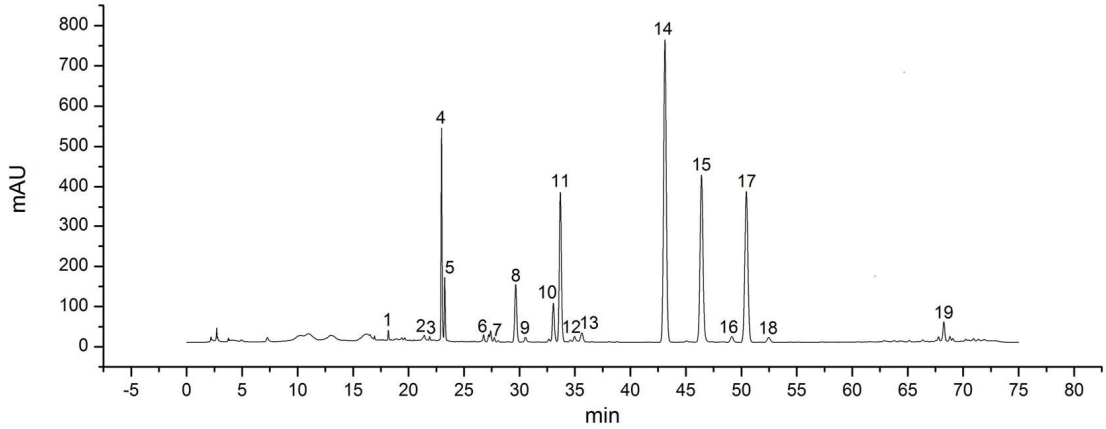

Figure 2: Characteristic fingerprint of 18 batches of Baizhi samples

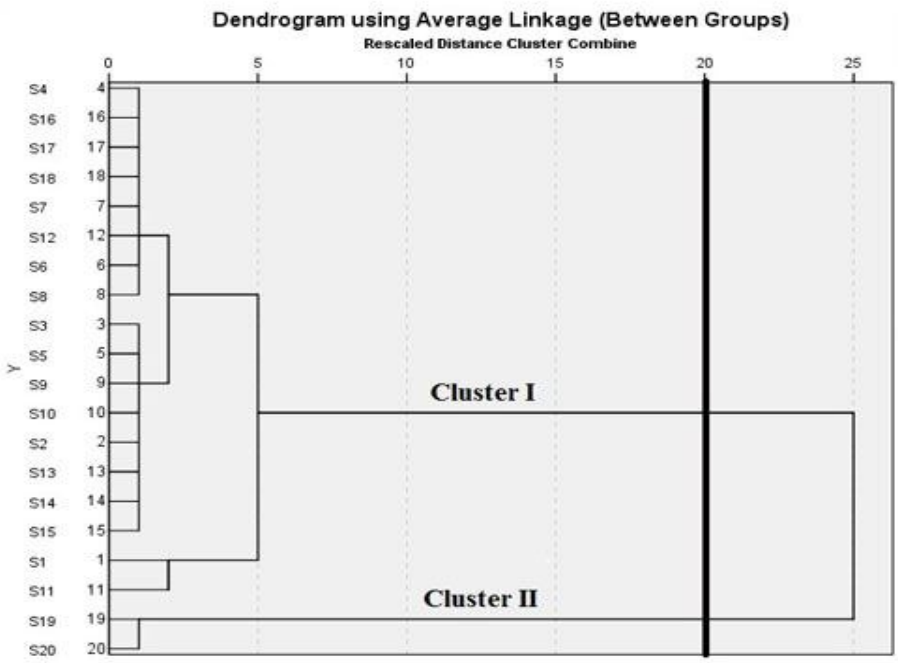

Figure 3: HCA of 20 batches of Baizhi samples

Hierarchical clustering analysis (HCA)

Nineteen common peak areas of 20 batches of Baizhi samples, processed with different sulfur fumigation time and dosages, were imported into SPSS 19.0 for HCA. The result was presented in Figure 3.

Table 3: Similarities of chromatograms of 18 samples, compared with the S1 chromatogram

No.	Similarity	No.	Similarity
S1	1.000	S10	0.951
S2	0.988	S11	0.977
S3	0.993	S12	0.914
S4	0.912	S13	0.966
S5	0.902	S14	0.969
S6	0.865	S15	0.967
S7	0.858	S16	0.911
S8	0.857	S17	0.880
S9	0.941	S18	0.901

Principal component analysis (PCA)

Nineteen common peak areas of 20 batches of Baizhi samples, processed with different sulfur fumigation time and dosages, were imported into SPSS 19.0 for PCA. The results of KMO and Bartlett's test suggested that the data could meet the technological requirement of PCA. Four components were extracted, used to replace the original data, by defining Eigen value and cumulative contribution rate. The results (Table 4) show that the contribution of PC1 was maximal among four components, which could represent the main original information and its formula was described in Eq 1 in which BXi stands for standardized variable of i variable. The coefficients of five peaks [peak 8 (isopimpinellin), 9, 11 (oxypeucedanin), 14 (imperatorin) and 15 (phellopterin)] were higher than 0.90, and these indicated that the changes of the five peak areas were the main change among samples. The five peak areas are listed in Table 5.

$$PC1 = 0.757BX_1 - 0.098BX_2 + 0.596BX_3 + 0.692BX_4 + 0.662BX_5 + 0.860BX_6 + 0.659BX_7 + 0.975BX_8 + 0.917BX_9 + 0.890BX_{10} + 0.934BX_{11} - 0.146BX_{12} + 0.739BX_{13} + 0.956BX_{14} + 0.921BX_{15} + 0.754BX_{16} + 0.798BX_{17} + 0.005BX_{18} - 0.733BX_1 \ldots\ldots (1)$$

DISCUSSION

In this work, chromatographic conditions, sample preparation process and HPLC-DAD method were optimized and validated to achieve a good separation of chemical constituents in Baizhi. Nineteen common peaks obtained by HPLC fingerprint analysis of Baizhi samples were used to analyze the influence of different sulfur fumigation time and dosages on chemical constituents' changes of Baizhi by SA, HCA and PCA. In addition, the 10 peaks of 19 common peaks were identified by standard substances.

The results of SA (Table 3) showed that the difference of similarities among 18 batches of Baizhi samples, processed with different sulfur fumigation time and dosages, were rather small, indicating that the influences of different sulfur fumigation time and dosages on the chemical

Table 4: Total variance explained

Component	Initial Eigen value			Extraction Sums of Squared Loading		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
PC1	10.556	55.560	55.560	10.556	55.560	55.560
PC2	3.740	19.685	75.245	3.740	19.685	75.245
PC3	1.301	6.848	82.093	1.301	6.848	82.093
PC4	1.035	5.446	87.539	1.035	5.446	87.539

Table 5: The changes of main peak areas with different levels of sulfur-fumigated Baizhi (n = 5)

No	Area Peak 8	No	Area Peak 9	No	Area Peak 11	No	Area Peak 14	No	Area Peak 15
S19	4946.7±89.6	S19	774.8±15.5	S19	28741.2±542.1	S20	24672.4±482.1	S19	14203.0±276.5
S20	3761.7±77.2	S20	555.5±11.3	S20	28131.2±536.8	S19	24158.0±472.5	S20	11578.1±241.2
S1	3457.4±69.5*	S1	370.7±8.1*	S1	11247.0±235.4*	S1	20691.4±413.5*	S11	9843.8±197.2*
S11	2718.9±56.8*	S11	232.0±4.9*	S3	8505.6±180.5*	S11	19591.3±392.6*	S1	9540.8±189.5*
S5	2648.1±48.7*	S3	230.7±4.6*	S5	7199.0±140.2*	S10	14772.1±285.4*	S13	9218.6±183.6*
S3	2511.5±50.3*	S13	184.2±3.7*	S11	5690.7±113.2*	S9	14743.7±294.7*	S2	8939.2±179.4*
S15	2483.6±49.2*	S5	171.8±3.4*	S15	4704.2±95.6*	S2	14609.2±291.5*	S9	7849.2±156.4*
S2	1883.6±37.1*	S12	158.1±2.9*	S13	4472.8±89.3*	S14	13590.1±271.8*	S15	7793.6±152.1*
S9	1882.7±38.9*	S2	156.0±3.1*	S14	3669.0±76.2*	S3	13505.6±269.3*	S3	7768.1±162.3*
S10	1804.0±35.8*	S18	148.4±2.8*	S2	3197.8±64.1*	S5	13315.2±266.5*	S7	7643.7±157.8*
S14	1756.6±34.2*	S7	144.8±2.9*	S10	2220.3±45.2*	S13	12810.6±254.2*	S12	7510.6±151.9*
S13	1569.7±28.9*	S14	123.7±2.5*	S9	1705.2±34.2*	S15	12561.2±251.6*	S14	7436.5±148.7*
S18	1540.7±30.3*	S8	122.2±2.6*	S16	915.3±19.5*	S12	12081.4±236.4*	S5	7425.9±148.7*
S12	1252.3±25.4*	S17	120.8±2.4*	S18	899.0±18.1*	S18	10768.9±215.6*	S10	6765.9±135.6*
S16	1082.2±20.9*	S15	118.3±2.3*	S4	883.5±16.5*	S16	9474.2±192.4*	S16	6177.2±125.8*
S17	1052.6±20.5*	S10	111.6±2.1*	S12	812.5±11.4*	S4	9126.8±189.6*	S4	5944.2±117.5*
S4	1044.1±19.6*	S9	106.3±2.3*	S17	573.3±12.1*	S7	9101.3±185.0*	S17	5903.5±118.1*
S8	872.8±17.6*	S16	103.8±1.9*	S6	276.6±5.8*	S17	8345.8±166.2*	S18	5774.4±115.4*
S7	833.9±15.9*	S4	94.3±1.7*	S7	205.7±4.8*	S8	6408.3±118.9*	S8	5237.8±104.8*
S6	453.8±10.1*	S6	39.0±1.1*	S8	28.0±0.6*	S6	4693.4±90.5*	S6	4375.1±89.2*

***Note**: * = $p < 0.05$, compared with no sulfur-fumigated samples (S19 and S20)*

composition of Baizhi were similar. In addition, the results of HCA (Figure 3) suggested that the 20 batches of samples could be classified into two clusters when Euclid Distance was 20.

Cluster I included 18 samples processed with different sulfur fumigation time and dosages and Cluster II contained two samples without sulfur fumigation (S19 and S20). It implied that the content of chemical constituents of Baizhi could be significantly decreased by sulfur fumigation ($p < 0.05$) though sulfur fumigation time and dosages were in low levels, 25 g (sulphur)/10 kg (Baizhi) (Sample S1) and 2 h (Sample S9). PCA had further verified the above viewpoint, and the influences of sulfur fumigation on the chemical composition of Baizhi were positively related to sulfur fumigation time and dosages according to the change of the main five peak areas (peak 8, 9, 11, 14 and 15).

CONCLUSION

The findings of this study indicate that the chemical constituents of Baizhi could be significantly decreased by sulfur fumigation even at low sulfur fumigation time and doses, suggesting that the sulfur fumigation is not a desirable method for field processing of Baizhi. It is therefore necessary to explore new field processing procedures that would not only improve the appearance of TCM but also extend the storage time of TCM, as well as prevent a reduction of its effective constituents in Baizhi.

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