DOI: 10.15806/j.issn.2311-8571.2016.0034 World J Tradit Chin Med 2017; 3(1): 16–21

Original Article

A precise and specific method for quick determination of sulfur fumigation for moutan cortex

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ABSTRACT

Objective: Establish a quick, precise and specific method to determine whether the moutan cortex obtained from market is processed with sulfur; provide a reliable method for the scientific evaluation.

Methods: Three methods, including acid-base titration method, high-performance liquid-chromatography (HPLC) method, and ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry (UHPLC-TOF-MS) method were used to detect whether 30 batches of samples were fumigated with sulfur.

Results: The results of three methods were substantially the same. Fifteen batches were identified to have been processed with sulfur fumigation, while others were not.

Conclusions: The HPLC method was found to be most appropriate for the determination of sulfur fumigation for moutan cortex.

Key words: Moutan cortex, Sulfur fumigation, Paeoniflorin, Paeoniflorin sulfonate, HPLC method
Received 16 August 2016; Accept 5 December 2016

Introduction

Moutan cortex (Mudanpi in Chinese), the root bark of *Paeonia suffruticosa* Andrews (Paeoniaceae)[1], is a well-known traditional Chinese medicine (TCM) for treating various diseases[2]. It is collected in autumn, removed from rootlets and soil, stripped off the root bark, and dried in the sun; or scraped off the coarse bark, removed from woody part and dried in the sun. It has been recorded in Chinese Pharmacopoeia with functions to clear heat, cool blood and active blood to resolve stasis[1]. Based on modern pharmacological studies, moutan cortex has shown the anti-microbial, anti-hypertensive, sedative, anti-convulsant, anti-oxidant[3], anti-alcoholic steatohepatitis[4] and anti-acute hepatotoxicity[5] effects. The main chemical components of moutan cortex are phenols, phenolic glycosides, monoterpenes and monoterpene glycosides[6]. Glycosides, such as paeoniflorin and benzoylpaeoniflorin, have shown good pharmacological effects on reducing blood viscosity, dilating blood vessels, improving microcirculation, and anti-oxidation[7].

Sulfur fumigation is historically and commonly used in the origin processing of TCMs for pest controlling, better appearance and the prevention of deterioration and decay[8]. However, residue of sulfur dioxide is harmful to human health and has caused common awareness in global. According to modern reports, it can irritate eyes and respiratory tract, cause stomach upset, diarrhea, nausea, and other adverse reactions[9-12]. There are even other problems related to moutan cortex. For example, the content of paeoniflorin (Figure 1A) which is the main component decreases while it transformed into paeoniflorin sulfonate (Figure 1B) during sulfur fumigation[13-16].

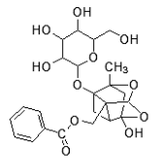
In order to assure the safety and the effectiveness of drug use, moutan cortex should not be fumigated with sulfur. The determination of the residue of sulfur dioxide in moutan cortex caused by sulfur fumigated is of great importance. Acid-base titration is a commonly-used method for the determination of the residue of sulfur dioxide. It is the first method to determine the residue of sulfur dioxide in Chinese Pharmacopoeia, 2015 version. However complex apparatus, difficult and time-consuming operations, and unspecific result made it not an ideal method for the determination. Therefore, a HPLC method using paeoniflorin sulfonate as the indicator, and an ultra-performance liquid chromatography coupled with a time-of-flight mass spectrometry (UHPLC-TOF-MS) method focused on the determination of paeoniflorin sulfonate and benzoylpaeoniflorin sulfonate were developed in our laboratory. Three methods were used and compared for distinguishing the sulfur fumigation of 30 batches moutan cortex. Different samples were determined and consistent results were obtained.

Experimental section

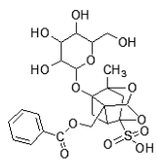
1. Instruments and reagents

1.1 Instruments

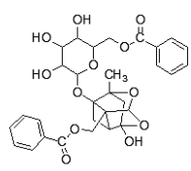
Electronic balance (Mettler Toledo, Shanghai); Ultrasonic machine (Elma P180H, Germany); Agilent-1260 HPLC



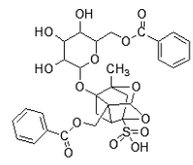
A Paeoniflorin
Formula: $C_{23}H_{28}O_{11}$
Molecular weight: 480.1632



B Paeoniflorin Sulfonate
Formula: $C_{23}H_{28}O_{13}S$
Molecular weight: 544.1251



C Benzoylpaeoniflorin
Formula: $C_{30}H_{32}O_{12}$
Molecular weight: 548.1894



D Benzoylpaeoniflorin Sulfonate
Formula: $C_{30}H_{32}O_{14}S$
Molecular weight: 648.1513

Figure 1. The structures of (A) paeoniflorin, (B) paeoniflorin sulfonate, (C) benzoylpaeoniflorin, (D) benzoylpaeoniflorin sulfonate.

system (Agilent Technologies, Palo Alto, CA, USA) was performed using a system including a G1315D diode-array detector, a G1311C binary pump, G1329B autosampler and G1316A column oven; UHPLC–MS analysis was performed using a G6230 LC/MS TOF system (Agilent Technologies, Palo Alto, CA, USA) with an Agilent-1290 UHPLC system coupled to a time-of-flight (TOF) tandem mass spectrometer via an electrospray ionization source (ESI). Analytical UHPLC was performed using a system including a diode-array detector, a G1312A binary pump, G1329 autosampler and G1316A column oven.

1.2 Reagents

Hydrogen peroxide solution (Sinopharm Chemical Reagent Co., Ltd, Shanghai), sodium hydroxide solution (Sinopharm Chemical Reagent Co., Ltd, Shanghai), hydrochloric acid solution (Sinopharm Chemical Reagent Co., Ltd, Shanghai), methanol (Sinopharm Chemical Reagent Co., Ltd, Shanghai), HPLC grade acetonitrile (Merck, USA); HPLC grade phosphoric acid (Tedia, USA), HPLC grade acetate acid (Tedia, USA), paeoniflorin sulfonate (Shanghai yuanye Bio-Technology Co., Ltd, Shanghai).

1.3 Samples

Sample sources of 30 batches moutan cortex were listed in Table 1.

2. Methods

2.1 Method A

The acid-base titration method (the first method for the determination of the residue of sulfur dioxide in Chinese Pharmacopoeia, 2015 version). More details were listed in the supporting information.

2.2 Method B

A HPLC method using paeoniflorin sulfonate as the indicator for sulfur fumigation analysis was developed in our laboratory.

Sample solution: Accurately weigh moutan cortex powder 0.2 g, add 5 ml 70% (v / v) methanol aqueous solution, with ultrasonic for 45 minutes (37 KHz, 50% power), then filter, and the successive filtrate was used as the sample solution.

Reference solution: Dissolve a quantity of paeoniflorin sulfonate, accurately weighed, in 70% (v / v) methanol aqueous solution to prepare a solution containing 1 mg of paeoniflorin sulfonate per ml.

The chromatographic separation was conducted on an Agilent Poroshell 120 SB-C18 column (4.6 mm × 150 mm, 2.7 μm) at 30°C. The elution was performed using water with 0.1% phosphoric (solvent A) and acetonitrile (solvent B) with a step gradient lasting 8 min. The eluting conditions were optimized as follows: 0/4/7/8 min, 10/10/14/15% B; the flow rate was 0.9 ml/min; detection wavelength was 232 nm; column temperature was set at 30°C; injection volume was 5 μL.

2.3 Method C

The UHPLC-TOF-MS method was developed in our laboratory.

Paeoniflorin sulfonate and benzoylpaeoniflorin sulfonate (Figure 1D) are two products of paeoniflorin during sulfur fumigation. According to reports, paeoniflorin decreases while it transformed into paeoniflorin sulfonate during sulfur fumigation. And benzoylpaeoniflorin (Figure 1C) was another peony glycoside content in moutan cortex which would transform into benzoylpaeoniflorin sulfonate (Figure 1D) after sulfur fumigation. Both of them were used as indicators for the sulfur fumigation analysis of the 30 batches moutan cortex by the UHPLC-TOF-MS method.

The sample solution and reference solution were the same as that in method B.

The chromatographic separation was conducted on an Agilent Eclipse Plus C18 RRHD column (2.1 mm × 150 mm, 1.8 μm). The elution was performed using 0.1% acetate acid (solvent A) and acetonitrile (solvent B) with a step gradient lasting 5 min. The eluting conditions were optimized as

Table 1. Sample source.

NO.	origin	NO.	origin
MC01	Tongling Anhui	MC16	Anhui
MC02	Bozhou Anhui	MC17	Bozhou Anhui
MC03	Tongling Anhui	MC18	Bozhou Anhui
MC04	Tongling Anhui	MC19	Bozhou Anhui
MC05	Tongling Anhui	MC20	Tongling Anhui
MC06	Bozhou Anhui	MC21	Anhui
MC07	Anhui	MC22	Bozhou Anhui
MC08	Tongling Anhui	MC23	Chongqing
MC09	Tongling Anhui	MC24	Shandong
MC10	Anhui	MC25	Anhui
MC11	Tongling Anhui	MC26	Anhui
MC12	Sichuan	MC27	Anhui
MC13	Shangluo Shanxi	MC28	Bozhou Anhui
MC14	Anhui	MC29	Anhui
MC15	Anhui	MC30	Anhui

Table 2. The results of three methods.

Batches	Method A SO_2	Method B PS	Method C PS	Method C BPS	Batches	Method A SO_2	Method B PS	Method C PS	Method C BPS
MC01	-	-	-	-	MC16	+	+	+	+
MC02	-	-	-	-	MC17	+	+	+	+
MC03	-	-	-	-	MC18	+	+	+	+
MC04	-	-	-	-	MC19	+	+	+	+
MC05	-	-	-	-	MC20	+	+	+	+
MC06	-	-	-	-	MC21	+	+	+	+
MC07	-	-	-	-	MC22	+	+	+	+
MC08	-	-	-	-	MC23	+	+	+	+
MC09	-	-	-	-	MC24	+	+	+	+
MC10	-	-	+	-	MC25	+	+	+	+
MC11	-	-	+	-	MC26	+	+	+	+
MC12	-	-	+	-	MC27	+	+	+	+
MC13	-	-	+	-	MC28	+	+	+	+
MC14	-	-	+	-	MC29	+	+	+	+
MC15	-	-	+	-	MC30	+	+	+	+

(PS stands for paeoniflorin sulfonate, BPS stands for benzoylpaeoniflorin sulfonate)

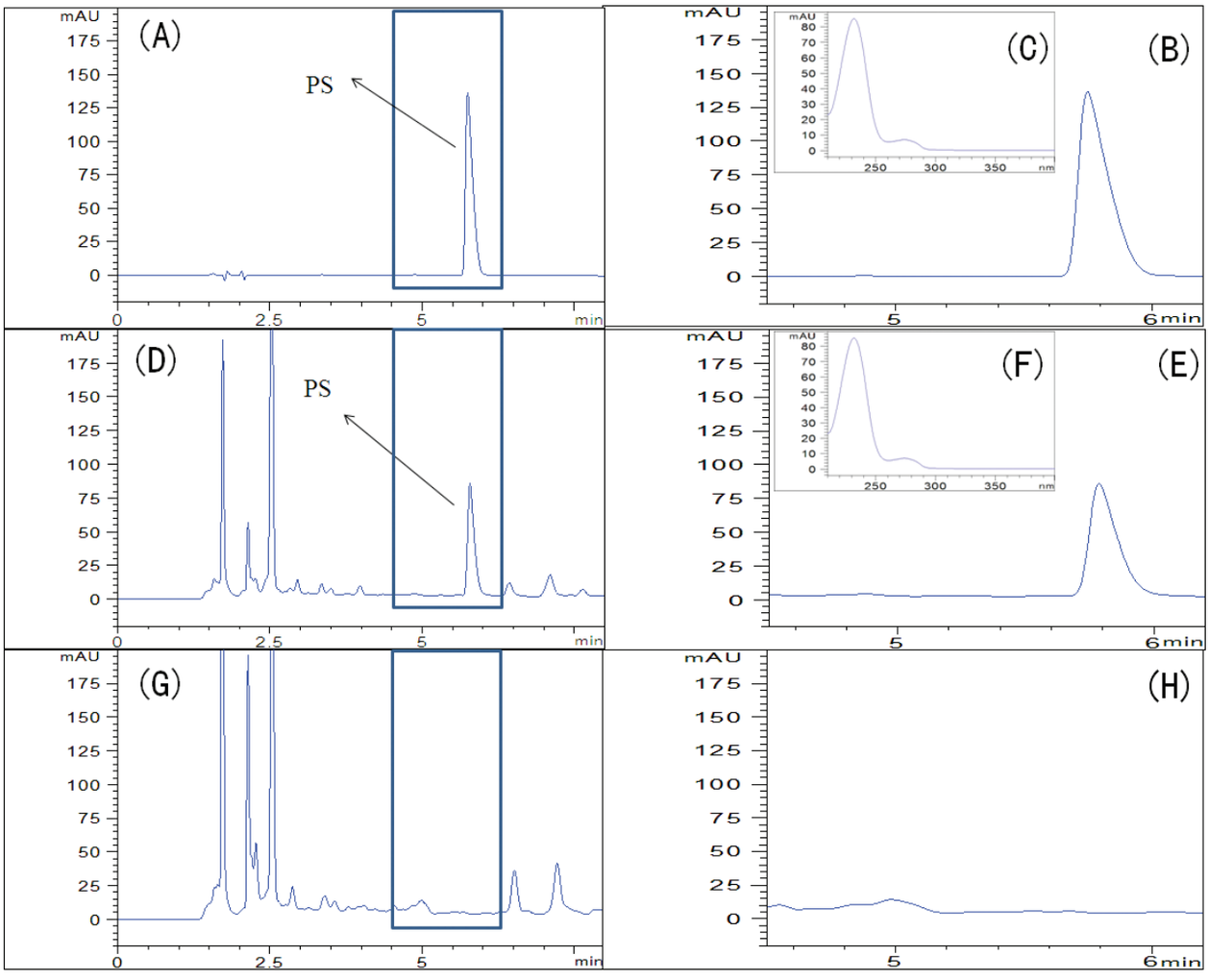


Figure 2. Chromatograms of (A) PS (paeoniflorin sulfonate), (B) the enlarged view of PS and (C) its spectrum, (D) MC16, (E) the enlarged view of MC16 and (F) its spectrum, (G) MC01, (H) the enlarged view of MC01 (blocks represent the enlarged regions).

follows: 0/1/3.5/5 min, 13/40/60/80%B, flow rate was 0.3 ml/min; detection wavelength was set at 232 nm; column temperature was 30°C; injection volume was 1 μL.

The Dual AJS ESI source was operated with a nebulizer pressure of 45 psi, while the drying gas was delivered at a flow rate of 8 L/min at 320°C. The sheath gas was delivered at a flow rate of 12 L/min at 350°C. Nozzle voltage was 1000 V. The capillary voltage, skimmer voltage, and fragmentor voltage were set at 3500 V, 65 V, and 170 V respectively. In the negative ionization mode, a solution containing ions of m/z 112.9855 and 1033.9881 were used as reference.

2.4 Ethical approval

The conducted research is not related to either human or animals use.

Results

1. The results of three methods (Table 2)

2. Method A

For MC01~15, the color of solutions remained the same. The color of sample solution (MC16~30) changed from yellow to red, which indicated the existence of sulfur dioxide in those batches. In the titration process, at least one drop of titrate (sodium hydroxide solution, 0.05 mL) would be used, which stands for 3.2 ppm of SO_2. So the limit of detection was determined as 3.2 ppm.

3. Method B

The results of method B were shown in figure 2 and 3. A peak with identical retention time and spectrum (Figure 2A, 2B and 2C) to paeoniflorin sulfonate was shown in the chromatograms of MC16~30, but not in those of MC01-15 (Figure 3). The results were fully consistent with method A. Limits of detections of method B: dilute the reference solution of paeoniflorin sulfonate to a certain concentration, making the instrument signal to noise ratio about 3 (corresponding concentration of paeoniflorin sulfonate was 0.03 ppm). The limit of detection was determined as 0.03 ppm, which indicated that method B was much more sensitive than method A.

4. Method C

Considering benzoylpaeoniflorin (Figure 1C) was another peony glycoside in moutan cortex which would transform into benzoylpaeoniflorin sulfonate (Figure 1D) after sulfur fumigation. In this study, both paeoniflorin sulfonate and benzoylpaeoniflorin sulfonate were screened in 30 batches crude drugs by method C. The content of benzoylpaeoniflorin in moutan cortex is very low, and the reference solution of benzoylpaeoniflorin sulfonate is hard to obtain. So the

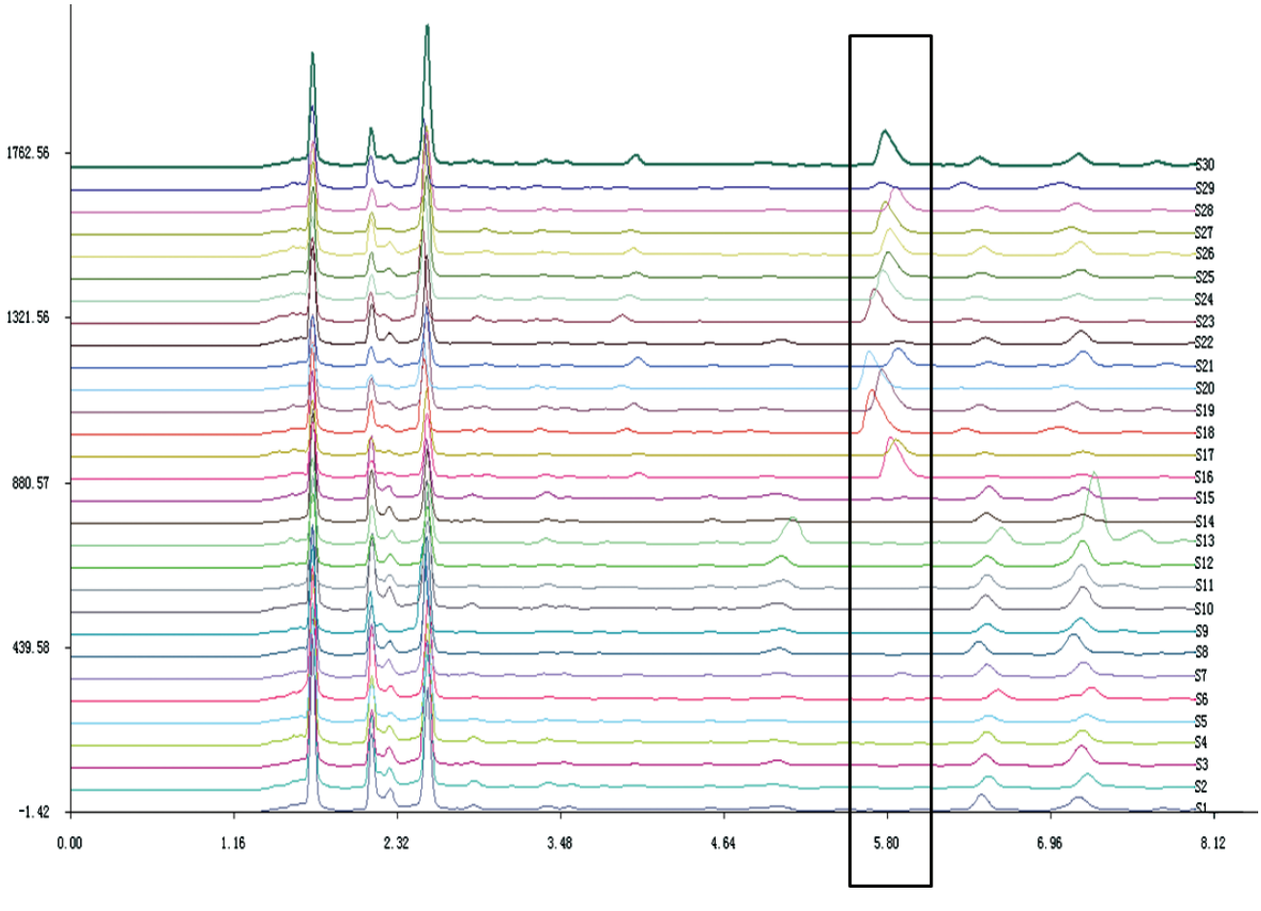


Figure 3. Chromatograms of S01~S30 (MC01~MC30).

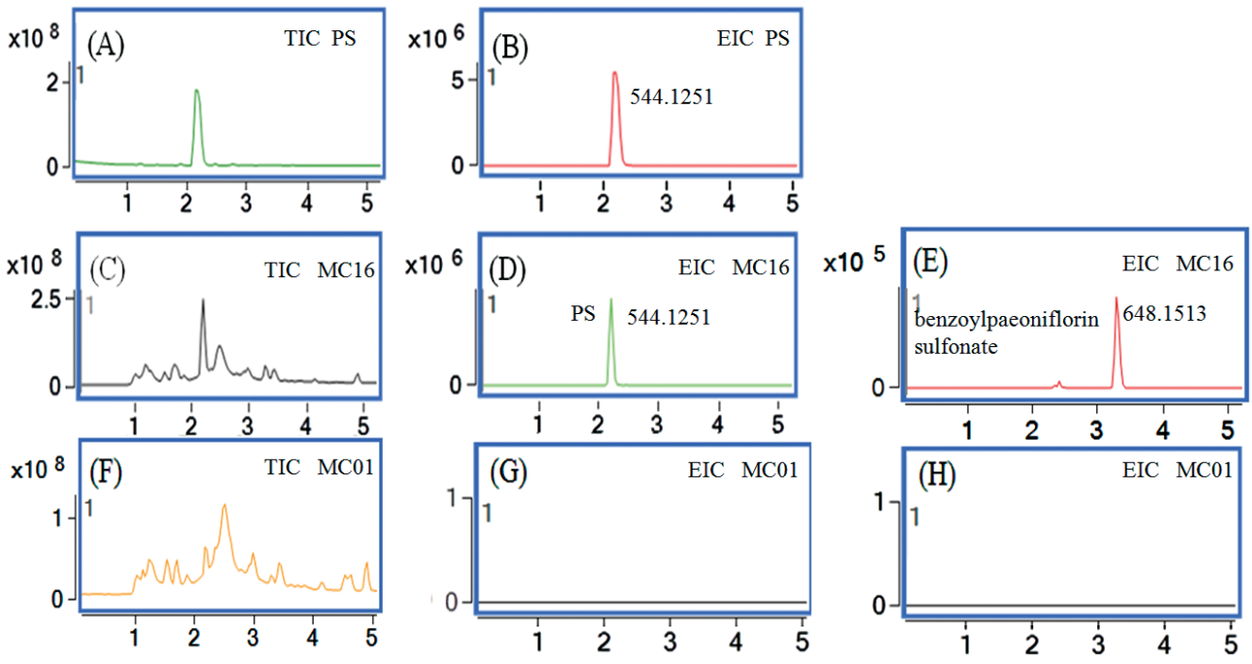


Figure 4. The total ion chromatograms of (A) PS (paeoniflorin sulfonate), (C) MC16, (F) MC01, the extracted ion chromatograms of (B) 544.1251 of PS, (D) 544.1251 of MC16, (E) 648.1513 of MC01, (G) 544.1251 of MC16, (H) 648.1513 of MC01.

molecular weight was chosen to match it. The results of method C were shown in figure 4. MC16 contained paeoniflorin sulfonate and benzoylpaeoniflorin sulfonate, while MC01 didn't. The results (Table 2) showed that twenty one batches (MC10-MC30) contained the peak of paeoniflorin sulfonate and nine batches (MC01-MC09) didn't. It was not consistent with the previous two methods. It was found that MC16-MC30 samples contained benzoylpaeoniflorin sulfonate and others not, which was also consistent with the previous two methods. The limit of detections of method C: dilute the reference solution of paeoniflorin sulfonate to a certain concentration, making the instrument could not detect it (corresponding concentration of paeoniflorin sulfonate was 0.015 ppm). The limit of detection was determined as 0.015 ppm. Method C was proved to be more accurate than the other two methods with higher sensitivity and the lower limit of detection.

The results of three methods were substantially the same. It was also proved that paeoniflorin sulfonatecould be used as an characterization indicator to distinguish the sulfured moutan cortex.

Discussion

Method B is more sensitive to detect the residue of sulfur dioxide of moutan cortex after sulfur fumigation than acid-base titration method. Acid-base titration method takes about two hours, and requires a special all-glass distillation apparatus. It is time-consuming and complicated, not easy to operate and not suitable for large batches' testing. In our study, an extract HPLC method was developed to distinguish the sulfured moutan cortex within 8 minutes which was suitable for mass tests. In order to confirm the accuracy of the method of high-performance liquid chromatography, ultra performance liquid chromatography tandem time of flight mass spectrometry was adopted to determine the 30 batches moutan cortex. Paeoniflorin sulfonate was qualitatively analyzed in 30 batches crude drugs. Although ultra-performance liquid chromatography tandem time of flight mass spectrometry is accurate and much quicker, its equipment is too expensive and not suitable for extensive promotion. So we recommend the use of high-performance liquid chromatography to determine whether the moutan cortex was sulfured.

Conclusion

Method A is the traditional method in Chinese Pharmacopoeia. It is time-consuming and requires complex apparatus. Method B is a newly developed method which has advantages of simple operation, short time and good repeatability. Meanwhile, method C is a more accurate method, but its machines are expensive and difficult to maintain. Considering the factors of time, manpower and material, method B is more superior to the other two methods. We recommend the method B to determine whether moutan cortex was sulfured.

Conflict of interest

The authors declare that there are no conflicts of interest.

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DOI: 10.15806/j.issn.2311-8571.2016.0034 World J Tradit Chin Med 2017; 3(1): 16–21

Original Article

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Supporting information

Method A:

Apparatus As the Figure S1, the apparatus consists of a 1000 ml double-neck round flask (A). an upright condensation (B), a graduated funnel (C), an import of nitrogen (D), and a gas export of sulfur dioxide (E). A magnetic mixer, heater, nitrogen source and gas flow meter are needed in addition.

Procedure Weigh accurately 10 g of the fine powder of crude drug or processed pieces in A, add 300 to 400 ml of water, open the reflux condenser switch, connect a gas-guide tube with E, insert the gas-guide tube into the bottom of a 100 ml of conical flask which contains 50 ml of 3% hydrogen peroxide solution as absorption solution. Before use, add 3 drops of methyl red ethanol solution indicator (2.5 mg/ml) to the absorption solution and titrate with 0.10 ml/L sodium hydroxide to yellow. Turn on nitrogen and adjust the gas flow to approximately 0.2 L/min by a flow meter; Open the piston of the graduated funnel(C). The hydrochloric acid solution (6 mol/L) was introduced into the distillation flask. Heat the two-necked flask of the solution to boiling immediately, and maintain micro-boiling; stop heating after 1.5 hours, After the solution was cooled, the solution was stirred on a magnetic stirrer and titrated with sodium hydroxide titration solution until the yellow color did not fade for 20 seconds. The titration results were correction with the blank experiment.

Content of sulfur dioxide residue in the test = ((A-B) *C*0.032*1000)/w Where A is the sodium hydroxide titration solution VS (ml) depleted of the test; B is the sodium hydroxide titration solution VS (ml) depleted of the blank; C is the concentration of sodium hydroxide titration solution VS (0.01 mol/L); W is the weight of the substances being examined (g); 0.032 is the weight of sulfur dioxide (g) equivalent to 1 ml of sodium hydroxide titration solution VS (1 mol/L).

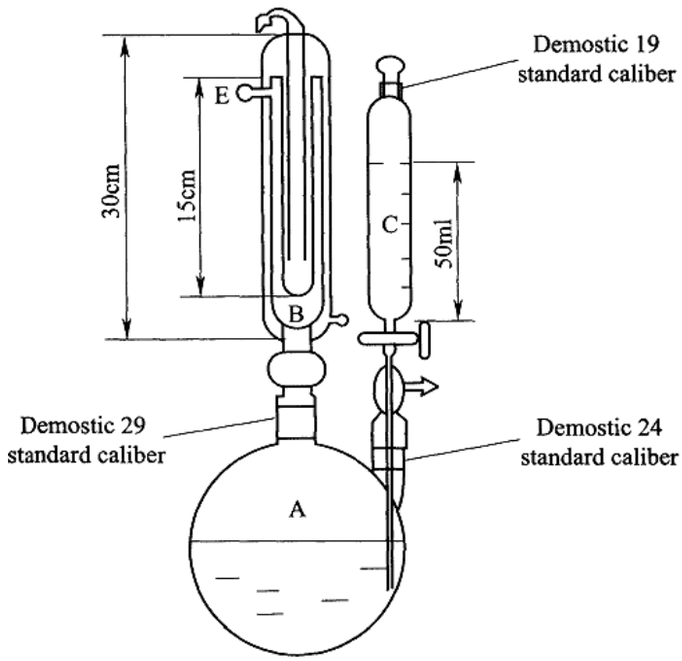

Figure S1. The apparatus of method A.