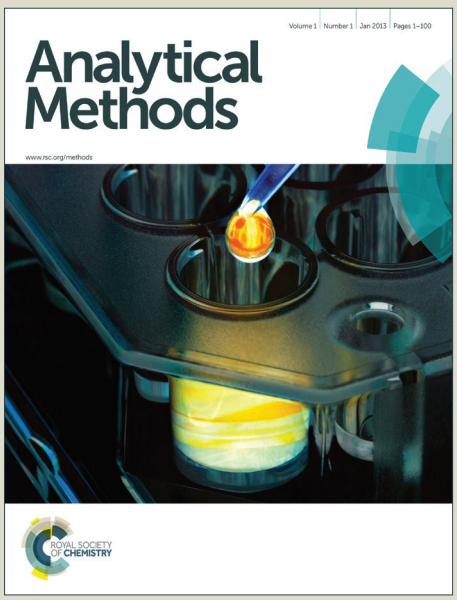
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

UPLC-QTOF-MS based metabolomics coupled with diagnostic ion exploration strategy for rapidly evaluating sulfur-fumigation caused holistic quality variation of medicinal herbs, Moutan Cortex as an example

Xiu-Yang Li[1,3¶], Jin-Di Xu[1,3¶], Jun Xu[4], Ming Kong[3], Shan-Shan Zhou[1], Qian Mao[3], Eric Brand[4], Hu-Biao Chen[4], Hong-Quan Liu[2*], Song-Lin Li[1,3*]

[1] Department of Pharmaceutical Analysis, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, People's Republic of China

[2] Department of Neurology, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, People's Republic of China

[3] Department of Metabolomics, Jiangsu Province Academy of Traditional Chinese Medicine, Nanjing , People's Republic of China

[4] School of Chinese Medicine, Hong Kong Baptist University, Hong Kong

*Corresponding authors:

E-mail: songlinli64@126.com (S L) or sunyu9186@sina.com (H L)

[¶]These authors contributed equally to this work.

Abstract

In the present study, a new strategy using UPLC-QTOF-MS based metabolomics coupled with diagnostic ion exploration for rapidly evaluating sulfur-fumigation caused holistic quality variation of medicinal herbs was proposed and validated by employing Moutan Cortex (MC), a commonly-used traditional Chinese medicinal herb, as an example. First, the UPLC-QTOF-MS data of MC and sulfur-fumigated MC (S-MC) were subjected to unsupervised segregation principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA), three chemical markers in S-MC was rapidly found and structurally elucidated to be pinane monoterpene glucosides sulfonates; Then, after exploring the MS fragmentation pattern of these chemical markers, a common sulfur-containing ion *m/z* 259 was selected as the diagnostic ion, and additional seven pinane monoterpene glucosides sulfonates were detected and identified in S-MS with the diagnostic ion extraction; Finally, the holistic quality variation of MC was further dissected by dynamic determination of these ten characteristic components at different durations of sulfur-fumigation. All the results indicated that sulfur-fumigation can induce chemical transformation of pinane monoterpene glucosides in MC, and the duration of sulfur-fumigation was a decisive factor in the holistic quality variation of S-MC, and that the proposed strategy should be applicable for rapid evaluation on sulfur-fumigation caused holistic quality variation of other medicinal herbs.

1. Introduction

Moutan Cortex (MC), the root bark of *Paeonia suffruticosa* Andr., is a common Chinese medicinal herb that is traditionally used for clearing heat, cooling the blood, promoting blood circulation, and eliminating stasis [1,2]. In modern clinical practice, MC is also employed for the treatment of rheumatoid arthritis and type-2 diabetes [3,4]. Accumulated modern research extensively demonstrates that MC possesses a wide range of pharmacological effects, including analgesic, anti-inflammatory, anti-platelet aggregation, anticancer, and cardiotonic activities [5-9]. Various types of constituents have been experimentally shown to be bioactive components of MC, in which pinane monoterpene glycosides are the most representatives [10-12].

In recent years, sulfur-fumigation has been widely employed in the post-harvest handling of many medicinal herbs; it serves as a low cost, high-efficiency approach to replace traditional processing methods such as sun-curing, and is used for sterilization, insect control, bleaching and to prevent corrosion [13,14]. However, sulfur-fumigation has been shown to alter bioactive components in the treated herbs by inducing chemical transformations, and consequently affects holistic quality of medicinal herbs [15-17]. The desirability of sulfur-fumigation for processing medicinal herbs thus remains controversial [13,18]. Nonetheless, sulfur-fumigated herbal materials, such as Angelicae Sinensis Radix [19,20], Ginseng Radix [21,22], Paeoniae Radix [23,24] and Codonopsis Radix [25], are still often found in herbal markets worldwide. To the best of our knowledge, no attention has been previously given to the effects of sulfur-fumigation on MC. Further research is necessary to

determine if and how sulfur-fumigation affects the holistic quality of MC, which should be significant for the safety and efficacy evaluation of sulfur-fumigated MC (S-MC).

Sulfur-fumigated medicinal herbs have been intensively evaluated for variations in their holistic quality. Nevertheless, unresolved questions remain regarding the methodologies employed in previous research. Frequently, quality evaluation is focused on assessing changes from sulfur-fumigation on the contents of several bioactive chemicals that are selected as markers [20,26,27]. However, it is well-known that medicinal herbs are characterized by many components, and their holistic attributes are derived from the actions or interactions of multiple components [28,29]. Therefore, this approach using quantitative determination of several chemical markers might be unable to reveal the holistic quality variations in some medicinal herbs. Furthermore, in most cases the mechanisms of the chemical transformations induced by sulfur-fumigation remain unknown. In recent years, with the development of analytical technologies and advancements in mass spectrometry in particular, chemical profiling has been widely adopted to characterize holistic quality variations in medicinal herbs caused by sulfur fumigation and other processing methods [17,21,25,30]. In these studies, the investigated herbs with and without sulfur-fumigation were analyzed and then intuitively and/or statistically compared using advanced liquid chromatography-mass spectrometry (LC-MS) tools. After that, both the original components and chemicals generated from the sulfur fumigation process were qualitatively identified one by one, and potential mechanisms of the

chemical transformations were individually considered. Although adequately comprehensive and thorough, the complete structural elucidation of whole chemicals is difficult and time-consuming, and therefore this method might be unsuitable for general and rapid analysis. Additionally, in these studies, characteristic chemical markers for the identification of sulfur-fumigated herbs are generally unavailable.

Metabolomics was initially proposed as a powerful approach for comprehensively profiling endogenous metabolites at a cellular or organ level to characterize the response of a living system to pathophysiological stimuli or genetic modification [31,32]. Currently, mass spectrometry-based metabolomics approaches are being successfully employed in many evaluations of the holistic quality of medicinal herbs [33,34]. Multiple advantages of metabolomics have been experimentally demonstrated, e.g. robust, comprehensive and sensitive [35]. On the other hand, ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry (UPLC-QTOF-MS) performs well in terms of providing abundant mass information with accurate mass measurement, and therefore is quite useful in the structural elucidation of unknown chemicals from medicinal herbs [36,37]. In this study, by using UPLC-QTOF-MS based metabolomics coupled with characteristic ion exploration, a novel and practical strategy was proposed for the rapid evaluation of holistic quality variations caused by the sulfur-fumigation of medicinal herbs, with MC as an example. First, the effects of sulfur-fumigation on the holistic quality of MC were comprehensively evaluated, and potential chemical markers for the identification of S-MC were statistically discovered by metabolomics analysis. Next,

sulfur-fumigation induced chemical transformations in S-MC were rapidly elucidated by diagnostic ion exploration. Finally, the holistic quality variations of S-MC were further dissected by dynamic determination of the chemical transformations that occurred at different durations of sulfur-fumigation.

2. Materials and Methods

2.1. Chemicals, reagents and materials

HPLC-grade acetonitrile was obtained from Merck Company (Darmstadt, Germany). Deionized water was purified using the Milli-Q system (Millipore, Bedford, MA, USA); formic acid was of MS grade and was obtained from ROE Company (Main.ST. Newark, USA). All other chemicals were of analytical grade and commercially available.

The reference compound paeoflorin sulfonate was purchased from Shanghai U-sea Biotech Co., Ltd. (Shanghai, China). The purity was higher than 98.0% as determined by HPLC analysis.

Fresh *Paeonia suffruticosa* samples were collected from the herbal garden of Nanjing University of Chinese Medicine. Fifteen batches of commercial MC were purchased from different pharmacies in China (Table 1). All samples were authenticated by Prof. S.L. Li to be the root bark of *P. suffruticosa* based on the monograph of MC documented in Chinese Pharmacopoeia (2010 version).

Table 1 Detection results of 15 batches of commercial MC samples

Code No.	Location	Collection Time	Results
JSPACM-DP-L-1	Beijing, China	07/21/2014	+
JSPACM-DP-L-2	Beijing, China	07/26/2014	-
JSPACM-DP-L-3	Beijing, China	07/27/2014	+
JSPACM-DP-L-4	Nanjing, China	07/28/2014	+
JSPACM-DP-L-5	Nanjing, China	07/30/2014	+
JSPACM-DP-L-6	Nanjing, China	07/30/2014	+
JSPACM-DP-L-7	Nanjing, China	07/31/2014	+
JSPACM-DP-L-8	Guangzhou, China	07/26/2014	+
JSPACM-DP-L-9	Guangzhou, China	07/26/2014	+
JSPACM-DP-L-10	Guangzhou, China	07/27/2014	+
JSPACM-DP-L-11	Guangzhou, China	07/28/2014	+
JSPACM-DP-L-12	Zhengzhou, China	07/28/2014	-
JSPACM-DP-L-13	Zhengzhou, China	07/29/2014	+
JSPACM-DP-L-14	Zhengzhou, China	07/30/2014	+
JSPACM-DP-L-15	Zhengzhou, China	07/31/2014	+

+: The three chemical markers (described in *Section 3.2*) were detectable;

−: The three chemical markers (described in *Section 3.2*) were undetectable

2.2. Sample preparation

The fresh MC samples were cut into slices with thickness of about 0.2 cm, and then separated into 5 portions. For preparing S-MC samples, a cylinder installation covered with plastic film was made to simulate the sulfur-fumigation conditions used by herbal farmers or wholesalers [23]. The installation was separated into upper and lower layers with a copper screen. MC slices moistened with water (1:1, w/v) were put onto the upper layer, while sulfur was put into a steel vessel and ignited, then moved into the lower layer. Four S-MC samples were accordingly prepared with fumigation durations of 2, 8, 16, and 26 h, respectively. After fumigation, the samples were dried at 50 ℃ for 2h. The MC sample was directly dried without sulfur

fumigation. All the samples were prepared in triplicate, packed in vacuum, and stored at 4 ℃ before use.

The prepared S-MC and MC samples were pulverized (40 mesh), accurately weighed (0.1 g) and then ultrasonic-extracted with 5 mL methanol (power 400 W, frequency 45 kHz) for 1 h. After that, the extracts were centrifuged at 9600 g for 10 min. The obtained supernatant was diluted to a proper concentration and filtered through a 0.22 μm filter for further analysis.

2.3. Liquid chromatography

UPLC was performed with a Waters ACQUITY UPLC system (Waters Corp., MA, USA), equipped with a binary solvent delivery system, auto-sampler, and a PDA detector. The separation was achieved on a Waters ACQUITY HSS T3 column (100 mm × 2.1 mm, 1.8 μm). The mobile phase consisted of (A) methanol containing 0.1 % (v/v) formic acid and (B) 0.1 % (v/v) aqueous formic acid. The UPLC elution condition was optimized as follows: 5 % A (0–1 min), 5–17 % A (1–4 min), 17–30 % A (4–9 min), 30–70 % A (9–16 min) and 70-100 % A (16–17 min), and the flow rate was 0.3 mL/min. The temperatures of the column and auto-sampler were maintained at 35 ℃ and 15 ℃, respectively. The injection volume of the standard and sample was 2.0 μL.

2.4. Mass Spectrometry

Mass spectrometry was performed on a Waters Synapt G2-S QTOF (Micro mass MS Technologies, Manchester, UK) equipped with electrospray ionization source operating at full scan mode. Data were monitored in negative ion mode. ESI

conditions were as follows: nebulization gas 6 bars at a temperature of 450 ℃, capillary voltage 2500 V, cone voltage 30 V, source temperature 100 ℃, desolvation temperature 400 ℃, cone gas flow 50 L/h, and desolvation gas flow 800 L/h. The QTOF acquisition rate was 0.2 s and the inter-scan delay was 0.02 s. During acquisition, alternating MS scans are collected at low (6 V) and high collision energy (30-60 V), providing precursor and fragment ions information, respectively. The mass spectrometer and UPLC system were controlled by MassLynx 4.1 software.

All MS data were acquired using the LockSpray to ensure mass accuracy and reproducibility. The molecular masses of the precursor ion and of product ions were accurately determined with leucine enkephalin (*m/z* 554.2615) in negative mode at the concentration of 200 pg/μL and the infusion flow rate was 5 μL/min. Centroided data were acquired for each sample from 80 to 1500 Da and dynamic range enhancement was applied in the MS experiment to ensure accurate mass measurement over a wide dynamic range.

2.5. Multivariate Statistical Analysis

MassLynx 4.1 software (Waters, Manchester, UK) was used to take the peak detection and alignment process for the acquired data. The method parameters were set as follows: retention time range of 2-17 min, mass range of 80-1500 Da, with a mass tolerance of 0.05 Da, the noise elimination level was set to 6.00, and the retention time tolerance was set to 0.2 min. No specific mass or adduct was excluded. Isotopic peaks were excluded in the analysis.

For data analysis, the intensity of each ion was normalized with respect to the total

ion count to generate a data matrix that consisted of the retention time, *m/z* value, and the normalized peak area. The multivariate data matrix was analyzed by EZinfo software 2.8 (Waters Corp., Milford, USA) and MetaboAnalyst 3.0. All the variables were mean-centered and paretoscaled prior to unsupervised segregation principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA).

3. Results and Discussion

3.1. Evaluation of holistic quality variations in S-MC

First, positive and negative ion modes for mass data acquiring were compared, and the negative modes was finally selected due to its superior sensitivity (Supplementary Fig. S1). To evaluate the holistic quality variations in MC caused by sulfur-fumigation, the obtained UPLC-MS data (*m/z*, t_R and ion intensity) from MC and S-MC samples at different durations of sulfur-fumigation (2 h, 8 h, 16 h and 26 h) were obtained by Pareto scaling and mean-centering, and were then subjected to PCA analysis. R^2X and Q^2 (cum) (EZinfo software 2.8) were used for evaluating the PCA model, and their acquired values were 0.743 and 0.703, respectively, indicating a good modeling quality of PCA. Two-component PCA model cumulatively accounted for 78.1% of variation (MetaboAnalyst 3.0). The PCA results were displayed as score plots to easily visualize the degree of gathering or dispersion among varied groups of samples by reducing the dimensionality of the complex data. As clearly seen in Fig. 1, the PCA score plots of the five groups of samples (MC and S-MC after 2, 8, 16, and 26 hours

of sulfur fumigation) were accordingly divided into five clusters and were well-separated with each other (all the samples in these groups fell well inside the 95% confidence interval). The diagram intuitively revealed that the holistic quality of MC was significantly changed by sulfur fumigation. Furthermore, interestingly, along with the increase in the duration of sulfur-fumigation, the clusters moved dynamically and were gradually away from the MC one. This tendency demonstrated that the duration of sulfur-fumigation should be a decisive factor in changes of the holistic quality of S-MC: within certain limits, the longer the duration of sulfur-fumigation, the more changes in holistic quality can be observed. The PCA loading plots (Fig. 1B) demonstrated the MS ions relative to the components that contribute to the difference among the five groups of samples.

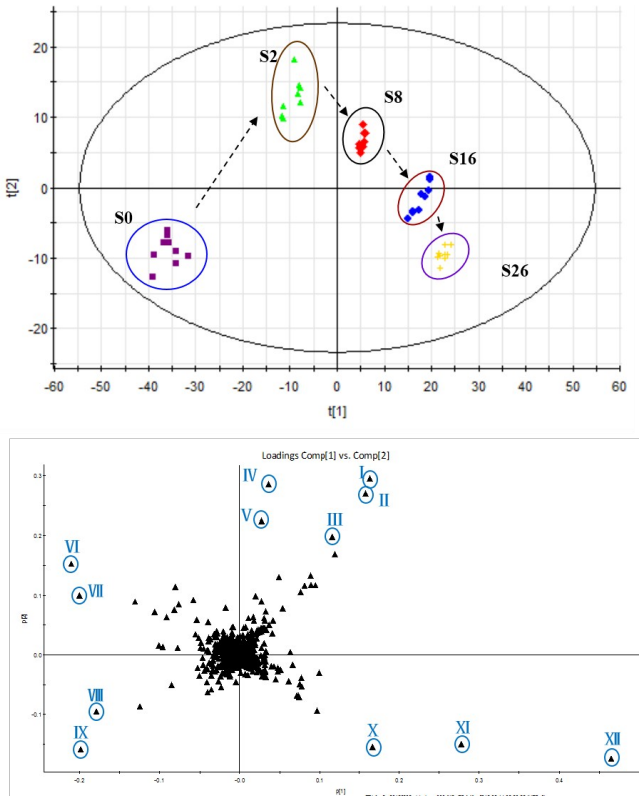


Fig.1 PCA score plot (A) and PCA loading plot (B) of Non-fumigated MC (S0) and Sulfur-fumigated MC for 2h, 8h, 16h, 26h (S2, S8, S16, S26) obtained using Pareto

scaling with mean centering in negative ion mode; Ⅰ (t_R 8.70 min, *m/z* 505.1564), Ⅱ (t_R 8.71 min, *m/z* 459.1505), Ⅲ (t_R 8.70 min, *m/z* 293.0875), Ⅳ (t_R 8.70 min, *m/z* 495.1266), Ⅴ (t_R 8.28 min, *m/z* 495.1267), Ⅵ (t_R 9.25 min, *m/z* 525.1612), Ⅶ (t_R 7.05 min, *m/z* 495.1504), Ⅷ (t_R 10.61 min, *m/z* 469.0509), Ⅸ (t_R 6.68 min, *m/z* 183.0298), Ⅹ (t_R 12.40 min, *m/z* 647.1430), Ⅺ (t_R 4.45 min, *m/z* 559.1131), Ⅻ (t_R 6.01 min, *m/z* 543.1178).

3.2. Exploration of chemical markers for S-MC identification

In order to explore potential chemical markers for the identification of S-MC, OPLS-DA was performed between MC and S-MC by S-plot analysis. Here the examples selected for illustration were the MC sample compared with the S-MC sample after 26 hours of sulfur fumigation. The observations fell within the Hotelling T2 (0.95) ellipse, where the model fit parameters were 0.999 of R^2Y (cum) and 0.998 of Q^2Y (cum) (EZinfo software 2.8), indicating that the OPLS-DA model established in this study owned well fitness and predictability. In the S-plot, each point represented an ion t_R-*m/z* pair and the points at the two ends of "S" that most contributed to the observed separation were selected as the potential chemical markers for the two groups, respectively. As shown in Fig. 2, **a** (t_R 6.01 min, *m/z* 543.1178), **b** (t_R 4.45 min, *m/z* 559.1131), **c** (t_R 8.71 min, *m/z* 459.1505), **d** (t_R 8.70 min, *m/z* 505.1564), **e** (t_R 12.40 min, *m/z* 647.1433), **f** (t_R 8.28 min, *m/z* 505.1560), **g** (t_R 8.70 min, *m/z* 293.0875), **h** (t_R 8.6488 min, *m/z* 373.1136) were the first eight ions from S-MC that successively contributed most to the S-MC differentiation from MC. Among them, ions **c**, **d**, **f**, **g** and **h** were detectable in both S-MC and MC, but ions **a**,

b and **e** were exclusive to S-MC. Ions **a**, **b** and **e** related components were therefore selected as chemical markers for the differentiation of S-MC from MC.

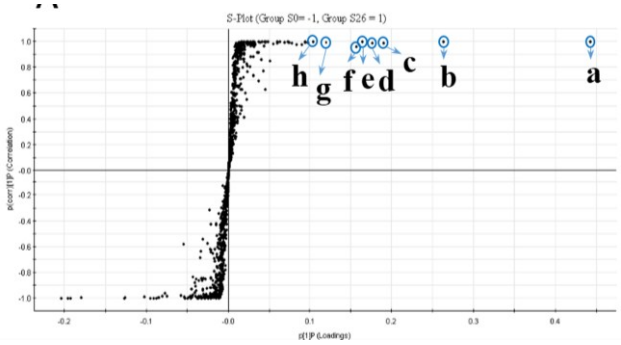


Fig. 2 S-plots of OPLS-DA between MC and S-MC (26 h). Ion **a** (t_R 6.01 min, *m/z* 543.1178) belongs to paeoflorin sulfonate; Ion **b** (t_R 4.45 min, *m/z* 559.1131,) belongs to oxypaeoflorin sulfonate; Ion **c** (t_R 8.71 min, *m/z* 459.1505), ion **d** (t_R 8.70 min, *m/z* 505.1560) and ion **g** (t_R 8.70 min, *m/z* 293.0875) belong to Paeonolide; Ion **e** (t_R 12.40 min, *m/z* 647.1433) belongs to benzoylpaeoflorin sulfonate; Ion **f** (t_R 8.28 min, *m/z* 505.1560) belongs to Apiopaeonoside; Ion **h** (t_R 8.6488 min, *m/z* 373.1136) was not identified.

To investigate commercially available MC herbal materials, fifteen batches of MC samples were randomly collected from different pharmacies, and screened using the newly-discovered chemical markers. Unexpectedly, the three chemical markers could be detected in thirteen of the fifteen batches (Table 1), which indicates that sulfur fumigation is widely employed for commercial MC processing.

3.3. Elucidation of sulfur-fumigation induced chemical transformations in MC

To further study the mechanisms of holistic quality variation in sulfur-fumigated MC, the sulfur fumigation-induced chemical transformations in MC were elucidated.

First, ion **a** related component, the most characteristic chemical marker of S-MC, was confirmed to be paeoniflorin sulfonate (compound **3**) by comparing the retention time, accurate masses and fragment ions with those of reference compound (Table 2 and Fig. 3). Paeoniflorin sulfonate is transformed from paeoniflorin, one of the main pinane monoterpene glycosides in MC (Fig. 4), by sulfur-fumigation [38,39]. In our previous studies, paeoniflorin sulfonate was also found in sulfur-fumigated Radix Paeoniae and its mass fragmentation pathway was preliminarily studied [24]. Here, the structural elucidation of paeoniflorin sulfonate based on mass fragments was further performed. As shown in Table 2 and Fig. 3, its mass spectra showed a deprotonated molecular ion $[M-H]^-$ at *m/z* 543.1178 in negative mode, suggesting that its empirical molecular formula was $C_{23}H_{28}O_{13}S$ and demonstrating an addition of SO_2 to paeoniflorin. The product ion at *m/z* 421.0805, loss of 122 Da from $[M-H]^-$, corresponding to the loss of a benzoic acid (122 Da) and the product ion at *m/z* 121.0292 further confirmed the assignment. Then, a product ion at *m/z* 259.0284 was obtained by subsequent loss of a glucosyl group. In addition, fragment ions at *m/z* 497.1111 displayed the loss of CH_2O_2 (46 Da) from the deprotonated molecular ion $[M-H]^-$ and its analogous fragment ions were also accordingly generated. The rationalization of the major mass fragments of paeoniflorin sulfonate was concluded in Fig. 4.

Table 2 Details of newly generated sulfonate derivatives identified in S-MC

Peak No.	tR (min)	Identity	Empirical formula	Mean measured mass (Da)	Theoretical exact mass (Da)	Erro (ppm)
1	4.45	Oxypaeoniflorin sulfonate	$C_{23}H_{27}O_{14}S[M-H]^-$	559.1131	559.1122	1.6
2	5.27	Mudanpioside E sulfonate	$C_{24}H_{29}O_{15}S[M-H]^-$	589.1217	589.1227	-1.7
3	6.01	Paeoniflorin sulfonate	$C_{23}H_{27}O_{13}S[M-H]^-$	543.1178	543.1172	1.1
4	7.14	Mudanpioside D sulfonate	$C_{24}H_{29}O_{14}S[M-H]^-$	573.1270	573.1278	-0.4
5	7.59	Galloyloxypaeoniflorin sulfonate	$C_{30}H_{31}O_{18}S[M-H]^-$	711.1219	711.1231	-1.4
6	8.98	Galloyl paeoniflorin sulfonate	$C_{30}H_{31}O_{17}S[M-H]^-$	695.1284	695.1282	0.3
7	10.09	Mudanpioside H sulfonate	$C_{30}H_{31}O_{16}S[M-H]^-$	679.1318	679.1333	-2.2
8	11.15	Benzoyloxypaeoniflorin sulfonate	$C_{30}H_{31}O_{16}S[M-H]^-$	663.1373	663.1384	-1.7
9	11.43	Mudanpioside C sulfonate	$C_{30}H_{31}O_{16}S[M-H]^-$	663.1370	663.1384	-2.1
10	12.41	Benzoylpaeoniflorin sulfonate	$C_{30}H_{31}O_{14}S[M-H]^-$	647.1433	647.1435	-0.3

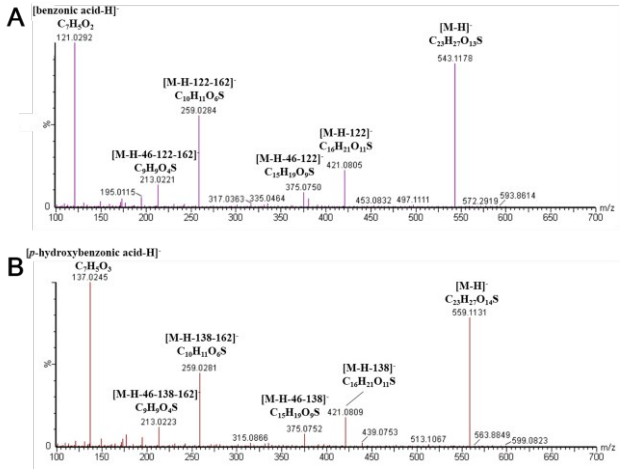


Fig. 3 High energy (30-60V) CID mass spectra of chemical markers in S-MC samples (S26 group) in negative mass mode: (A) Paeoniflorin sulfonate; (B) Oxypaeoniflorin sulfonate.

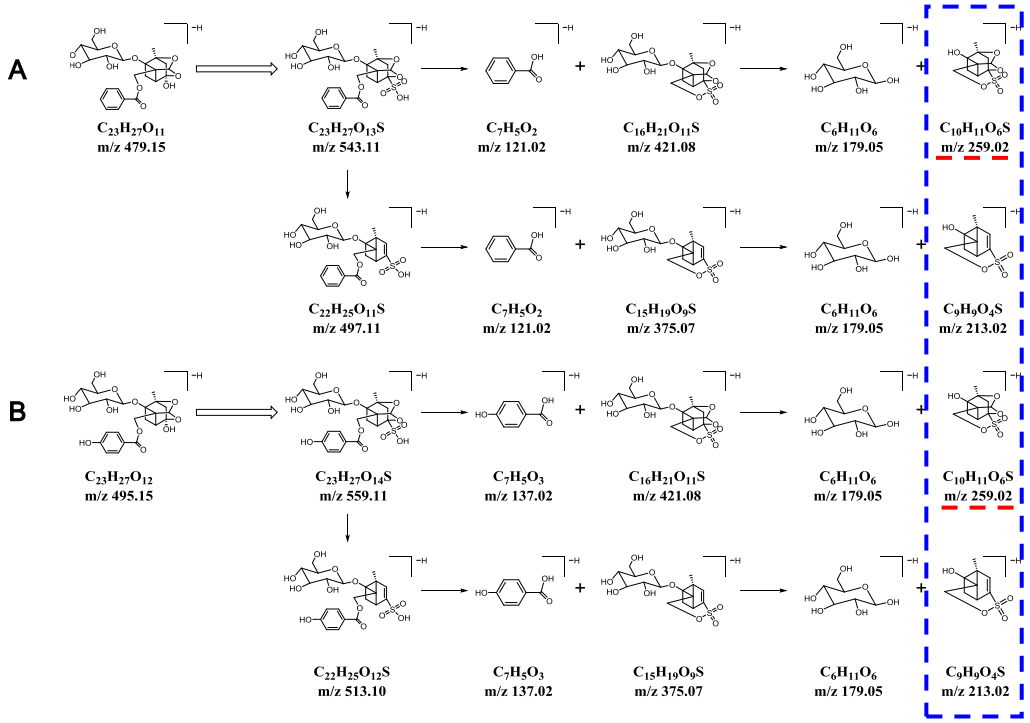


Fig. 4 Chemical transformation by sulfur-fumigation (⇨) and mass fragmentation pathways (→) proposed for (A) paeoniflorin sulfonate and (B) oxypaeoniflorin sulfonate.

Previous studies have demonstrated that paeoniflorin-like pinane monoterpene glycosides are widely present in MC as the main bioactive components. They normally possess a same "cage-like" pinane skeleton with different substituent groups, typically glucosyl and phenyl-containing groups [40,41]. Therefore it can be easily deduced that the sulfur fumigation-induced chemical transformation of paeoniflorin might also occur in other pinane monoterpene glycosides. In addition, the structure of paeoniflorin sulfonate suggests that the sulfur-fumigation induced reaction on this kind of chemical should happen at the hydroxyl group on the pinane skeleton rather than other substituents. And the mass fragmentation pathway of paeoniflorin sulfonate presented here shows that after successive loss of the substituents, the newly-generated sulfur-containing pinane skeleton was still stable and could not be

fragmented even with high collision energy. All these facts hinted that the diagnostic ions of the sulfur-containing pinane skeleton (*m/z* 259 and *m/z* 213) could be used for screening sulfonate derivatives of the other pinane monoterpene glycosides. Hence, in this study, *m/z* 259, with much more abundant ion intensity than *m/z* 213, was employed for extraction ion analysis. Interstingly, in addition to paeoniflorin sulfonate, nine more pinane monoterpene glucoside sulfonates were found in S-MC in the extraction ion chromatogram (*m/z* 259), two of which are the known chemical markers: ions **b** and **e** (Fig. 5). By matching the empirical molecular formula with that of the published known pinane monoterpene glucosides in MC [24,42,43], the nine chemicals were rapidly identified as oxypaeoniflorin sulfonate (**1**, ion **b**), mudanpioside E sulfonate (**2**), mudanpioside D sulfonate (**4**), galloyloxypaeoniflorin sulfonate (**5**), galloyl paeoniflorin sulfonate (**6**), mudanpioside H sulfonate (**7**), benzoyloxypaeoniflorin sulfonate (**8**), mudanpioside C sulfonate (**9**), and benzoylpaeoniflorin sulfonate (**10**, ion **c**), respectively (Fig. 6 and Table 2). Extraction ion chromatograms (EICs) and mass spectra for the identified compounds were shown in supplementary Fig. S2. The structural elucidation was described by taking oxypaeoniflorin sulfonate (**1**, ion **b**) as an example (Fig. 3). Similarly, the first diagnostic ion at *m/z* 259 was generated by consecutive neutral losses of a *p*-hydroxybenzonic acid (138 Da) and a glucosyl group (162 Da), while the other diagnostic ion at *m/z* 213 was produced by successive losses of CH_2O_2 (64 Da), *p*-hydroxybenzonic acid (138 Da), and a glucosyl group (162 Da). Thus the chemical was identified as oxypaeoniflorin sulfonate, and it was confirmed by the given

quasi-molecular ion at *m/z* 559.1131 $[M-H]^-$ (Fig. 3 and 4).

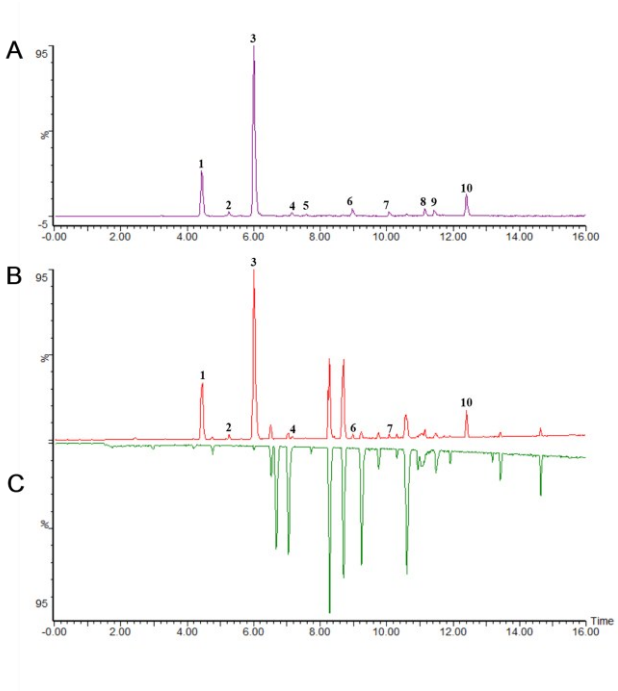
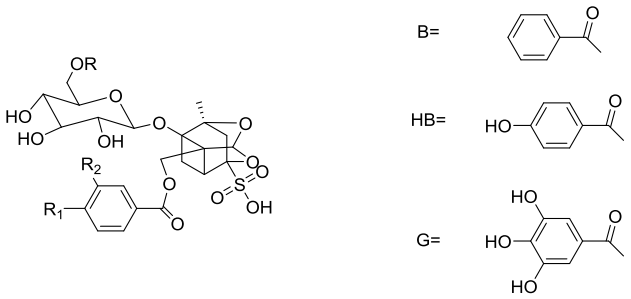


Fig. 5 Extraction ion (*m/z* 259) chromatogram (A) and base peak ion chromatogram (B) of the S-MC (S26) and base peak ion chromatogram (C) of the MC (S0).

1. Oxypaeoniflorin sulfonate, 2. Mudanpioside E sulfonate, 3. Paeoniflorin sulfonate, 4. Mudanpioside D sulfonate, 5. Galloyloxypaeoniflorin sulfonate, 6. Galloyl paeoniflorin sulfonate, 7. Mudanpioside H sulfonate, 8. Benzoyloxypaeoniflorin sulfonate, 9. Mudanpioside C sulfonate, 10. Benzoylpaeoniflorin sulfonate



No.	Compound	R	R_1	R_2
1	Oxypaeoniflorin sulfonate	H	OH	H
2	Mudanpioside E sulfonate	H	OH	OCH_3
3	Paeoniflorin sulfonate	H	H	H
4	Mudanpioside D sulfonate	H	OCH_3	H
5	Galloyloxypaeoniflorin sulfonate	G	OH	H
6	Galloyl paeoniflorin sulfonate	G	H	H
7	Mudanpioside H sulfonate	HB	OH	H
8	Benzoyloxypaeoniflorin sulfonate	B	OH	H
9	Mudanpioside C sulfonate	HB	H	H
10	Benzoylpaeoniflorin sulfonate	B	H	H

Fig. 6 Chemical structures of the ten identified pinane monoterpene glucoside sulfonates

In the base peak ion (BPI) chromatogram of S-MC, seven main peaks were newly generated compared with that of MC (Fig. 5). Interestingly, all these peaks were exactly the pinane monoterpene glucoside sulfonates detected in the extraction ion chromatogram (*m/z* 259) since they shared the same retention time and mass spectrometry information. The other three chamicals were not found in the BPI chromatogram of S-MC, possibly due to the insufficient sensitivity and selectivity of total ion analysis compared with extraction ion analysis. This fact indicated that pinane monoterpene glucoside sulfonates could be the main forms of sulfur-fumigation induced chemical transformations in S-MC, and should be largely responsible for the holistic quality variations in S-MC.

3.4. Dynamic determination of transformed chemicals in MC during sulfur-fumigation

We have verified that pinane monoterpene glucosides are the main bioactive chemicals in MC that are transformed by sulfur-fumigation. Therefore, to more illustrate the effects of the duration of sulfur fumigation on the holistic quality of MC,

the ten newly-generated sulfonate derivatives together with their corresponding prototypical pinane monoterpene glucosides were simultaneously and dynamically determined by extraction ion analysis at five time points (0, 2, 8, 16, 26 h) during the 26 h sulfur-fumigation. The results are provided in Fig. 7. The whole experimental procedure including sulfur-fumigation, ultrasonic extraction and LC-MS analysis was performed in triplicate, and the determined results were repeatable (RSD<7.69%). It is obvious that the prototypical pinane monoterpene glucosides decreased inordinately during this period. Meanwhile, their sulfonate derivatives increased accordingly. The results further confirmed our previous conclusion that the duration of sulfur fumigation plays an important role in the holistic quality variation of S-MC, and within the 26 h process of sulfur fumigation, the holistic quality of S-MC increasingly changed over time.

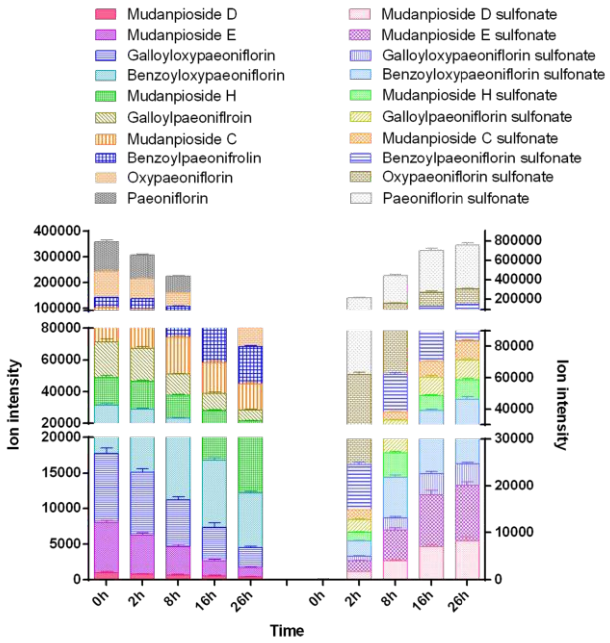


Fig. 7 Relative quantification of the ten pinane monoterpene glucoside sulfonates and its corresponding prototypical chemicals within 26 h sulfur-fumigation. All data with

three replicates were reported as mean ± standard deviation.

4. Conclusions

In this study, a novel strategy using an UPLC-QTOF-MS based metabolomics approach coupled with diagnostic ion exploration was employed for the rapid evaluation of holistic quality variations in MC due to sulfur fumigation. The experimental results suggested that sulfur-fumigation could significantly affect the holistic quality of MC by chemically transforming pinane monoterpene glucosides, the main bioactive components of MC, to their corresponding sulfonate derivatives. Among them, three pinane monoterpene glucoside sulfonates, namely paeoniflorin sulfonate, oxypaeoniflorin sulfonate and benzoylpaeoniflorin sulfonate, were statistically selected as chemical markers for the differentiation of S-MC from MC. Sulfur-containing ion *m/z* 259 could be used as the diagnostic ion to screen pinane monoterpene glucoside sulfonates in S-MC. The proposed approach was quiet efficient to reveal sulfur-fumigation effect on the drug chemical profile in an untargeted manner. Hopefully it will also be useful for evaluating other sulfur-fumigated medicinal herbs.

Acknowledgements

This study was financially supported by National High Technology Research and Development Plan of China (863 Plain) (2014AA022204), National Natural Science Foundation of China (No.81503245), The Administration of Traditional Chinese

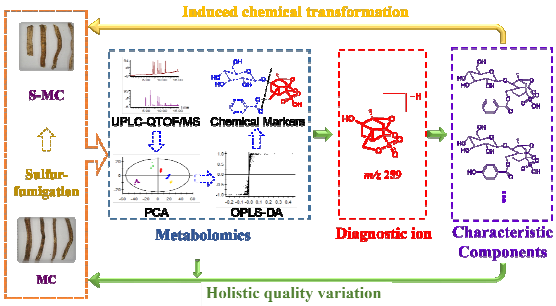
Medicine of Jiangsu Province (LZ13075), Jiangsu Province Six Talent Project (YY-007) and Jiangsu Branch of China Academy of Chinese Medical Science (JSBN1301).

References:

1. *The State Pharmacopoeia Commission of People's Republic of China*, China Medicial Science Press, Beijing, 2010, pp. 160-161.
2. *The Chinese Materia Medica*, ed. Z. Q. Lei, China Press of Traditional Chinese Medicine, Beijing, 2nd edn., 2007, vol. 9, ch. 4, pp. 146-147.
3. W. L. Tang, L. Jun and S. Y. Xu, *Chin. Pharmacol. Bull.*, 2002, **18**, 656-660.
4. D. T. Ha, T. N. Trung, T. T. Hien, T. T. Dao, N. Yim, T. M. Ngoc, W. K. Oh and K. Bae, *J. Ethnopharmacol.*, 2010, **131**, 417-424.
5. H. S. Chae, O. H. Kang, Y. S. Lee, J. G. Choi, Y. C. Oh, H. J. Jang, M. S. Kim, J. H. Kim, S. I. Jeong and D. Y. Kwon, *Am. J. Chin. Med.*, 2009, **37**, 181-194.
6. P. K. Fu, C. Yang, T. H. Tsai and C. L. Hsieh, *Phytomedicine.*, 2012, **19**, 1206-1215.
7. A. Hirai, T. Terano, T. Hamazaki, J. Sajiki, H. Saito, K. Tahara, Y. Tamura and A. Kumagai, *Thromb. Res.*, 1983, **31**, 29-40.
8. G. S. Oh, H. O. Pae, H. Oh, S. G. Hong, I. K. Kim, K. Y. Chai, Y. G. Yun, T. O. Kwon and H. T. Chung, *Cancer Lett.*, 2001, **174**, 17-24.
9. Y. Wang, L. Liu, C. Hu and Y. Cheng, *Biochem. Pharmacol.*, 2007, **74**, 415-424.
10. G. Chen, L. Zhang and Y. Zhu, *J. Pharm. Biomed. Anal.*, 2006, **41**, 129-134.
11. M. Yoshikawa, E. Uchida, A. Kawaguchi, I. Kitagawa and J. Yamahara, *Chem. Pharm. Bull.*, 1992, **40**, 2248-2250.
12. M. Wu and Z. Gu, *Evid. Based. Complement. Alternat. Med.*, 2009, **6**, 57-63.
13. W. L. T. Kan, B. Ma and G. Lin, *Front. Pharmacol.*, 2011, **2**, 84.
14. J. J. Liu, X. Liu, S. L. Li, B. C. Cai, H. Cai, *Chin. Tradit. Herbal. Drugs.*, 2010, **41**, 1403-1406.
15. Y. Cheng, C. Peng, F. Wen and H. Zhang, *J. Ethnopharmacol.*, 2010, **129**, 167-173.
16. J. Y. X. Zhan, P. Yao, C. W. C. Bi, K. Y. Z. Zheng, W. L. Zhang, J. P. Chen, T. T. X. Dong, Z. R. Su and K. W. K. Tsim, *Phytomedicine.*, 2014, **21**, 1318-1324.
17. A. L. Guo, L. M. Chen, Y. M. Wang, X. Q. Liu, Q. W. Zhang, H. M. Gao, Z. M. Wang, W. Xiao and Z. Z. Wang, *Molecules.*, 2014, **19**, 16640-16655.
18. X. Jiang, L. F. Huang, S. H. Zheng and S. L. Chen, *Phytomedicine.*, 2013, **20**, 97-105.
19. Y. J. Lou, H. Cai, X. Liu, G. Cao, S. C. Tu, S. L. Li, X. Q. Ma, K. M. Qin and B. C. Cai, *Pharmacogn. Mag.*, 2014, **10**, S189-S197.
20. W. L. Wei and L. F. Huang, *Molecules.*, 2015, **20**, 4681-4694.
21. S. L. Li, H. Shen, L. Y. Zhu, J. Xu, X. B. Jia, H. M. Zhang, G. Lin, H. Cai, B. C. Cai, S. L. Chen and H. X. Xu, *J. Chromatogr. A.*, 2012, **1231**, 31-45.
22. H. M. Zhang, S. L. Li, H. Zhang, Y. Wang, Z. L. Zhao, S. L. Chen and H. X. Xu, *J. Pharm. Biome. Anal.*, 2012, **62**, 258-273.
23. M. Kong, H. H. Liu, J. Xu, C. R. Wang, M. Lu, X. N. Wang, Y. B. Li and S. L. Li, *J. Pharm. Biomed. Anal.*, 2014, **98**, 424-433.
24. S. L. Li, J. Z. Song, F. F. K. Choi, C. F. Qiao, Y. Zhou, Q. B. Han and H. X. Xu, *J. Pharm. Biomed. Anal.*, 2009, **49**, 253-266.
25. X. Q. Ma, A. K. M. Leung, C. L. Chan, T. Su, W. D. Li, S. M. Li, D. W. F. Fong

and Z. L. Yu, *Analyst.*, 2014, **139**, 505-516.
26. G. Fan, R. Deng, L. Zhou, X. Meng, T. Kuang, X. Lai, J. Zhang and Y. Zhang, *Phytochem. Anal.*, 2012, **23**, 299-307.
27. X. Jin, L. Y. Zhu, H. Shen, J. Xu, S. L. Li, X. B. Jia, H. Cai, B. C. Cai and R. Yan, *Food Chem.*, 2012, **135**, 1141-1147.
28. L. Wang, G. B. Zhou, P. Liu, J. H. Song, Y. Liang, X. J. Yan, F. Xu, B. S. Wang, J. H. Mao, Z. X. Shen, S. J. Chen and Z. Chen, *Proc. Natl. Acad. Sci. USA.*, 2008, **105**, 4826-4831.
29. A. S. Attele, J. A. Wu and C. S. Yuan, *Biochem. Pharmacol.*, 1999, **58**, 1685-1693.
30. H. Cai, G. Cao, L. Li, X. Liu, X. Q. Ma, S. C. Tu, Y. J. Lou, K. M. Qin, S. L. Li and B. C. Cai, *Molecules.*, 2013, **18**, 1368-1382.
31. X. J. Wang, A. H. Zhang, Y. Han, P. Wang, H. Sun, G. C. Song, T. W. Dong, Y. Yuan, X. X. Yuan, M. Zhang, N. Xie, H. Zhang, H. Dong and W. Dong, *Mol. Cell. Proteomics.*, 2012, **11**, 370-380.
32. W. B. Dunn and D. I. Ellis, *Trac-Trend. Anal. Chem.*, 2005, **24**, 285-294.
33. X. Lu, X. J. Zhao, C. M. Bai, C. X. Zhao, G. Lu and G. M. Xu, *J. Chromatogr. B.*, 2008, **866**, 64-76.
34. H. G. Gika, G. A. Theodoridis, R. S. Plumb and I. D. Wilson, *J. Pharm. Biomed. Anal.*, 2014, **87**, 12-25.
35. E. Cubero-Leon, R. Penalver and A. Maquet, *Food. Res. Int.*, 2014, **60**, 95-107.
36. A. Nordstrom, G. O'Maille, C. Qin and G. Siuzdak, *Anal. Chem.*, 2006, **78**, 3289-3295.
37. G. Theodoridis, H. G. Gika and I. D. Wilson, *Trac-Trend. Anal. Chem.*, 2008, **27**, 251-260.
38. P. Y. Hayes, R. Lehmann, K. Penman, W. Kitching and J. J. De Voss, *Tetrahedron Lett.*, 2005, **46**, 2615-2618.
39. Q. Wang, R. X. Liu, H. Z. Guo, Z. N. Zhu, K. S. Bi and D. A. Guo, *Chin. J. Chin. Mater. Med.*, 2006, **31**, 1418-1421.
40. C. N. Xiao, M. Wu, Y. Y. Chen, Y. J. Zhang, X. F. Zhao and X. H. Zheng, *Phytochem. Anal.*, 2015, **26**, 86-93.
41. Y. Song, Q. He, P. Li and Y. Y. Cheng, *J. Sep. Sci.*, 2008, **31**, 64-70.
42. S. J. Xu, L. Yang, X. Zeng, M. Zhang and Z. T. Wang, *Rapid. Commun. Mass. Spectrom.*, 2006, **20**, 3275-3288.
43. J. Zhang, H. Cai, G. Cao, X. Liu, C. Wen and Y. Fan, *Evid. Based. Complement. Alternat. Med.*, 2013, DOI: 10.1155/2013/763213.

Graphical abstract:



Textual abstract:

A novel strategy using UPLC-QTOF-MS based metabolomics coupled with diagnostic ion exploration for rapidly evaluating sulfur-fumigation caused holistic quality variation of medicinal herbs is proposed.