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1. Introduction

Sulfur fumigation is a traditional storage method for Chinese herbal medicines (CHMs). It was first applied to the processing of *Dioscoreae rhizoma*, and has been widely misused in various CHMs in the last two decades, such as in the processing of *Chrysanthemi* Flos, *Gastrodia Rhizoma*, *Radix Paeoniae Alba* and *Lonicera japonica* Flos (FLJ).¹⁻³ It plays an important role in the production and post-harvest handling of CHMs due to its usage in moisturizing, bleaching, retaining freshness and killing parasites.⁴⁻⁶ However, sulfur fumigation induces significant chemical transformations in inherent herbal constituents, resulting in alterations in the bioactivities, pharmacokinetics and toxicities of CHMs.^{7,8} Besides, it often leads to the production in CHMs of excessive sulfur dioxide (SO₂), sulfates, sulfites, heavy metal residues and other detrimental exogenous

A proposed protocol based on integrative metabonomics analysis for the rapid detection and mechanistic understanding of sulfur fumigation of Chinese herbal medicines[†]

Dai Shengyun,^{ab} Wang Yuqi,^a Wang Fei,^{ac} Mei Xiaodan^a and Zhang Jiayu^b*^{de}

In the current work, *Lonicera japonica* Flos (FLJ) was selected as a model Chinese herbal medicine (CHM) and a protocol was proposed for the rapid detection of sulfur-fumigated (SF) CHMs. A multiple metabonomics analysis was conducted using HPLC, NIR spectroscopy and a UHPLC-LTQ-Orbitrap mass spectrometer. First, the group discriminatory potential of each technique was respectively investigated based on PCA. Then, the effect of mid-level metabonomics data fusion on sample spatial distribution was evaluated based on data obtained using the above three technologies. Furthermore, based on the acquired HRMS data, 76 markers discriminating SF from non-sulfur-fumigated (NSF) CHMs were observed and 49 of them were eventually characterized. Moreover, NIR absorptions of 18 sulfur-containing markers were identified to be in close correlation with the discriminatory NIR wavebands. In conclusion, the proposed protocol based on integrative metabonomics analysis that we established for the rapid detection and mechanistic explanation of the sulfur fumigation of CHMs was able to achieve variable selection, enhance group separation and reveal the intrinsic mechanism of the sulfur fumigation of CHMs.

materials, which exhibit harmful potential toxicities or side effects to human health.⁹⁻¹¹

Although the use of sulfur fumigation has been officially restricted in China since 2005,¹² some illicit herbal farmers and wholesalers still misuse sulfur fumigation during the postharvest handling and storage of CHMs. Moreover, SO₂ residue-based detection standards formulated by many countries and organizations are often ineffective at evaluating the degree of sulfur fumigation because of the high volatility of SO₂. Current studies mainly focus on total SO₂ residues and neglect the transformations of inherent herbal constituents and corresponding mechanisms.^{13–16} Therefore, the development of rapid and sensitive approaches based on stable quality-markers (Q-markers), such as sulfur-containing derivatives, to discriminate sulfur-fumigated (SF) CHMs from non-sulfur-fumigated (NSF) CHMs is urgently needed.¹⁷

Integrative omics combining and interpreting data from multiple sources have already been adopted to successfully elucidate the mechanisms of human diseases, such as diabetes, obesity and schizophrenia.^{18,19} Besides, integrative omics analysis has been used to characterize genes in the context of the molecular pathophysiology of the disease and its interacting genes and pathways.^{20,21} Likewise, multi-omics data collected using various detection technologies such as liquid chromatography combined with mass spectrometry (LC-MS), highperformance liquid chromatography (HPLC) and near infrared

^aSchool of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 102488, China

^bNational Institute of Food and Drug Control, Beijing 100050, China

^cDepartment of Pharmacy, People Hospital of Peking University, Beijing 100044, China ^dBeijing Research Institute of Chinese Medicine, Beijing University of Chinese Medicine, Beijing 100029, China. E-mail: zhangjiayu0615@163.com

^eSchool of Pharmacy, Binzhou Medical University, Yantai 264003, China

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(NIR) spectroscopy have been used for the screening and identification of Q-markers for the analysis of CHMs.^{22,23} Of these technologies, HPLC retains the practicality and principles of LC, while increasing the overall interlaced attributes of sensitivity and resolution, MS has emerged as a powerful tool for quantitative and qualitative analysis of the complex components in CHMs, and NIR spectroscopy is a very rapid and alternative nondestructive method that shows electromagnetic absorption signals in the NIR region associated with specific chemical structures and that can be assigned to specific chemical functional groups and molecular structures. Nevertheless, although each technique has its own powerful capabilities for specific issues, any data set obtained by one single technique cannot capture the complexity of the overall system. Thus, integrative metabonomics analysis based on multiple levels of data fusion and correlation combines the information provided by various analytical technologies so as to achieve much better statistical predictions and interpretations than those obtained from any individual technique.

FLJ, also known as Jin Yin Hua in Chinese, is one of the most well-known CHMs. It is derived from the dried buds or flowers of Lonicera japonica Thunb. and contains various biological ingredients such as organic acids, flavonoids and iridoid glycosides.24-27 Pharmacological investigations indicated that FLJ displays various pharmacological activities, such as hepatoprotective, cytoprotective, anti-microbial, anti-oxidative, anti-viral and anti-inflammatory activities.28-30 FLJ is also used in many food products, such as FLJ tea, a well-known health drink that has been highly praised for thousands of years for clearing away heat and toxic materials and treating exogenous pathogenic wind-heat.31 However, in the last two decades, sulfur fumigation has been frequently misused in post-harvest handling during the drying and storage of FLJ. Therefore, we used FLJ as a study case to present a proposal for a protocol based on integrative metabonomics analysis in order to clarify the inherent chemical transformations of CHMs and to classify the CHMs based on these transformations. SF and non-sulfurfumigated (NSF) FLJ along with organic acids, flavonoids and iridoid glycosides were used to verify the effectiveness of the established strategy.

2. Materials and methods

2.1 Chemicals, reagents and herbal materials

A total of 22 batches of authenticated NSF FLJ samples were collected from several different areas in China (Table S1[†]). All of their identities were authenticated using morphological and histological methods to be the dried buds of *L. japonica* Thunb. according to the monograph of Chinese Pharmacopoeia (version 2015).³² The authenticated specimens were deposited in the Beijing Research Institute of Chinese Medicine, Beijing University of Chinese Medicine, China. Standard substances including 3-caffeoylquinic acid (3-CQA), 4-CQA, 5-CQA, 3,4-dicaffeoylquinic acid (3,4-DiCQA), 3,5-diCQA, 4,5-diCQA, lonicerin, secologanic acid, swertiamarin and luteolin 7-O-β-glucoside were purchased from Chengdu Bio-purify Phytochemicals Ltd (Sichuan, China) with their purities all greater than 98%

(Fig. S1 and Table S2†). HPLC-grade acetonitrile and methanol were provided by Fisher Scientific (Fisher, Fair Lawn, NJ, USA). Formic acid was provided by Aldrich (St. Louis, MO). All of the other chemicals were of analytical grade and obtained commercially from Beijing Chemical Works (Beijing, China). De-ionized water was purified using a Milli-Q Gradient A 10 System (Millipore, Billerica, MA). The 0.22 µm membranes were purchased from Xinjinghua Co. (Shanghai, China).

2.2 Sample preparation

2.2.1 SF FLJ herbal samples. Fifteen batches of SF FLJ samples were prepared following the modified procedures that were employed by farmers and illicit wholesalers.^{33,34} A total of 200 g of FLJ dried buds were wetted with water and allowed to stand for 0.5 h. Afterwards, a proper amount of sulfur powder was heated until it burned, and then the burning sulfur and wetted FLJ were carefully put into, respectively, the lower and upper layers of a desiccator. The desiccator was then kept closed for 12 h in order to achieve a sufficient sulfur fumigation. Meanwhile, the SF 5-CQA that was utilized for result validation was also prepared with this same method.

2.2.2 Reference solutions. A certain amount of each of 3-CQA, 4-CQA, 5-CQA, 3,4-CQA, 3,5-diCQA, 4,5-diCQA, lonicerin, secologanic acid, swertiamarin and luteolin 7-O- β -glucoside was respectively weighed accurately and then dissolved in methanol to obtain the mixed reference solutions (0.01 mg mL⁻¹). These reference solutions were stored at 4 °C prior to analysis.

2.2.3 FLJ sample solutions. FLJ powders ground and sieved through a 65 mesh sieve were soaked for 30 min. A total of 1.0 g of powder was accurately weighed and then extracted with 25 mL methanol/water (70 : 30, v/v) in an ultrasonic bath (40 kHz, Eima Ultrasonics Corp., Germany) for 30 min at room temperature. Then the same solvent was added to compensate for the lost weight during the extraction process. The methanol solution was subjected to centrifugation (10 000 g) for 10 minutes, and then filtered through a 0.22 μ m microporous membrane before being injected into an LC-MS system for analysis. To ensure the quality of the HPLC and LC-MS-based metabonomics data, pooled quality control (QC) samples were prepared by mixing equal amounts of 37 sample solutions.

NIR, HPLC-DAD and UHPLC-LTQ-Orbitrap MS data were collected from these samples. The conditions for the three methods are listed in the ESI.[†]

2.3 Primary metabonomics data processing

All of the data were subjected to principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to interpret the interrelationships between the samples. With respect to PLS-DA, samples were divided into a calibration set for modeling and a validation set for the established model evaluation. The prediction set consisted of samples that were not used for the calibration set.

The very high quantities of acquired UHPLC-LTQ-Orbitrap MS raw data were processed with an Xcalibur 2.1 workstation (Thermo Scientific, Germany). The normalization was

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accomplished using Sieve 2.1 software (Thermo Scientific, USA), which was specifically used to perform background subtraction, component detection and peak alignment. SIMCA-P+ 11.5 (Umetrics, Sweden) and Unscrambler 7.0 (CAMO, Norway) software were utilized to carry out the spectral pre-processing. PCA and PLS were conducted using Matlab version R2009a (The MathWorks, Inc., USA) with Statistical Toolbox and inhouse functions. The iToolbox utilized to run synergy interval partial least squares (siPLS) analysis algorithms was downloaded from http://www.models.kvl.dk/ for the NIR wavelength selection.

2.4 Integrative metabonomics analysis

2.4.1 Metabonomics data fusion. In order to integrate the information acquired from the different technologies, mid-level metabonomics data fusion was carried out to investigate the three obtained metabonomics data sets based on NIR, HPLC and LC-MS approaches. Much more attention was paid to the comparison between two methods, *i.e.*, metabonomics data fusion with or without variable selection. The first method specifically applied PCA to describe the metabonomics data and determine the data structure without variable selection. To overcome the shortage of differences in scores and eigenvalues, all of them were standardized and eigenvalues were converted into percentage of explained variance. Scores were then multiplied by eigenvalues, and matrices were combined (HPLC, NIR and HRMS). The second method was based on the selection of relevant variables for each analytical method using PLS-DA with variable selection, which was then investigated using PCA. In this way, the number of explanatory variables was markedly reduced, and the selected variables only described the primary differences between all of the samples.

2.4.2 Method validation. To ensure the reliability of the experimental results, the reference standard, 5-CQA, was obtained to perform the SF process. SF chlorogenic acid samples (0.5, 1.0, 2.0, 2.5 and 5.0 mg) were respectively weighed, and each sample was then thoroughly mixed with 5.0 mg of dextrin. Meanwhile, NSF chlorogenic acid samples were prepared in the same way to test the reliability of the results. These ten samples were analyzed using the same NIR and LC-MS methods.

3. Results and discussion

3.1 Basic data sets for the three technologies

Typical NIR, HPLC-DAD and total ion chromatogram (TIC) results of the representative SF and NSF samples are presented in Fig. S2.[†] After peak alignment and removal of the missing values, 90 features were finally obtained for the HPLC-based metabonomics analysis. Meanwhile, for each of 37 samples, the NIR and MS data sets included 1557 and 5000 variables, respectively.

3.2 Primary metabonomics data analysis

Primary data analysis results were obtained respectively from unsupervised PCA and supervised PLS-DA for the three different techniques (Table 1 and Fig. 1). As for PCA, SF and NSF FLJ samples were not explicitly clustered into two groups with regards to HPLC-DAD and NIR analysis (The preprocess method was SG9+2nd, and the results obtained from the other preprocess methods are illustrated in Fig. S3 and Table S3†). While for the LC-MS analysis results, the 37 SF and NSF samples did segregate into two distinct groups. Fig. 1A–C show plots of these scores for these three techniques, respectively. A distinct classification trend could be observed in the LC-MS score plot. However, the results for these samples were nevertheless scattered considerably, with that for sample number 16 attributed to the NSF group being located in the SF group, which indicated that some of the variation in the samples cannot be obtained from the PCA.

Therefore, PLS-DA was performed to improve the group separation. The PLS-DA model resulted in a clear separation of the SF and NSF samples for each of the three different technologies (Fig. 1D-F). As for the HPLC and LC-MS analyses, statistical models were considered to be statistically significant when the corresponding Q^2 -intercept values (-0.262 and -0.264) for the permutation model were negative. Meanwhile, the permuted R^2 values (0.624 and 0.439) were lower than the original R²-values (0.903 and 0.927). Additionally, analysis of variance of the cross-validated predictive residuals (CV-ANOVA) tests were performed to confirm that the SF and NSF groups discriminated by PLS-DA were significantly different. The common practice was to interpret a *p* value $(1.16 \times 10^{-5} \text{ and }$ 3.73×10^{-11}) dramatically lower than 0.05 as contributing a significant model. As for the NIR analysis (for which the preprocess method was SG9+2nd, and the results obtained from the other preprocess methods are shown in Fig. S4^{\dagger}), the Q^2 intercept value was lower than 0.5, indicating the poor predictive capability of PLS-DA here. The poor predictive capability was also verified by the finding of a positive Q^2 -intercept value from the permutation test. Therefore, the use of the NIR and HPLC technologies did not achieve a satisfactory classification based on PCA while the use of MS did so.

3.3 Integrative metabonomics analysis

3.3.1 Mid-level metabonomics data fusion analysis without variable selection. Data fusion is defined as the integration of data blocks from different analytical platforms into a single model so as to improve the capability of statistical prediction and facilitate interpretation.³⁵ Low-level, mid-level and high-level data fusion have been three commonly used strategies. As the most commonly used strategy, mid-level data fusion can either combine variables after a relevant selection procedure or concatenate latent variables extracted from different statistical methods. Herein, the mid-level metabonomics fusion of NIR, HPLC and MS data was investigated. PCA was respectively conducted with various combinations of these techniques, specifically NIR-HPLC, HPLC-MS, NIR-MS and NIR-HPLC-MS (Table 2).

Compared to the primary metabonomics analyses for HPLC and NIR alone, the principal factor total cumulative based on the results of the fusion of NIR and HPLC data was higher, with a value of 93.5%. However, the discrimination was still

Table 1 Primary data analysis results for the three techniques^a

Technique	PCA		PLS						
	Lvs	$R^2(X)$	Lvs	$R^2(Y)$	Q^2	Q ² -intercept	Permuted R^2 value	<i>p</i> -Value	
HPLC-DAD	3	35.7%	3	94%	63.5%	-0.262	0.624	$1.16 imes 10^{-5}$	
NIR	5	39.0%	3	97.2%	55.2%	0.504	0.504	0.037	
LC-MS	6	72.0%	2	94.9%	82.9%	-0.264	0.439	$3.73 imes10^{-11}$	

^{*i*} Lvs: the number of latent variables.

unsatisfactory. NSF and SF samples were dispersed in the threedimensional space, indicating that the key information about the discrimination between the two analyses might not be captured (Fig. 2A). HPLC-MS-, NIR-MS- and NIR-HPLC-MSbased metabonomics data fusion generated summarized principal factorial plane results that were similar to the result for MS analysis mentioned above. Besides, many more variables were in the MS data set than in the HPLC data set, and hence the information contained in the MS data set could cover up the limited information of HPLC to some extent. Therefore, the results from MS-HPLC metabonomics data fusion were similar to those from primary metabonomics based on the MS data set (Table 3).

3.3.2 Mid-level metabonomics data fusion analysis with variable selection. Neither primary data analysis nor data fusion of different analytical technologies without variables

selection could distinctly discriminate SF from NSF samples. This observation indicated that data fusion could not increase the classification capability, as the obtained results from metabonomics data fusion analysis were not improved greatly

 Table 2
 The results of the mid-level data fusion analysis for the three techniques

	PCA		PLS-PCA	1
Techniques	Lvs	$R^2(X)$	Lvs	$R^2(X)$
NIR-HPLC	5	93.5%	5	55.2%
HPLC-MS	5	71.4%	6	79.4%
NIR-MS	6	75.0%	5	66.9%
NIR-HPLC-MS	6	74.4%	5	66.7%



Fig. 1 The results of primary metabonomics data fusion analysis. (A–C) PCA for HPLC-DAD, NIR and LC-MS; (D–F) PLS-DA for HPLC-DAD, NIR and LC-MS.



Fig. 2 The results of mid-level metabonomics data fusion analysis. (A–D) Mid-level metabonomics data fusion analysis without variable selection for NIR-HPLC, HPLC-MS, NIR-MS and NIR-HPLC-MS. (E–H) Mid-level metabonomics data fusion analysis with variable selection for NIR-HPLC, HPLC-MS, NIR-MS and NIR-HPLC-MS.

when compared to those obtained from the primary data analysis.

Subsequently, we investigated the mid-level metabonomics data fusion with PLS-DA to improve the group separation. The results from NIR-HPLC, HPLC-MS, NIR-MS and NIR-HPLC-MS data fusions were respectively presented after preliminarily screening all of data acquired from an individual platform according to variable importance values (VIP > 1.0). The initial NIR, HPLC and MS data sets included 1557, 90, and 5000 variables, respectively. After the screening based on the VIP scores, 607, 27 and 1843 variables, respectively, were considered to be the most effective variables and hence retained for the subsequent discrimination. So now, a new PCA model could be constructed to enhance the group discrimination of SF and NSF samples based on the generated variables data set.

HPLC-NIR data fusion using the new PCA model yielded much better results than ever before (Fig. 2E), even though 37 batches of FLJ were not distinctly clustered into two groups. Meanwhile, the HPLC-MS data fusion generated much better results without any misclassification, while one misclassification was still found in the NIR-MS data fusion results (no. 16 was still far from the NSF group) (Fig. 2F and G).

Fig. 2H shows the results obtained from NIR-MS-HPLC metabonomics data fusion. Although no. 16 was not correctly allocated into the NSF group, the group discrimination potential was significantly improved when compared with those obtained from metabonomics data fusion without variable selection. Thus it could be seen from the results that

metabonomics data fusion with variable selection made greater improvements in class separation than did the metabonomics data fusion without variable selection.

3.3.3 Identification of the markers discriminating NSF and SF FLJ samples based on LC-MS metabonomics analysis. As LC-MS could make up for the drawbacks in the structural identification of the discriminatory markers, a UHPLC-LTQ-Orbitrap high-resolution mass spectrometer was employed to perform the discrimination of the SF and NSF FLJ samples. To obtain satisfactory group separation based on differential variables and precisely distinguish the discriminatory markers, we applied the VIP values to filter several variables that contributed to them. Meanwhile, S-plots and t-tests were also typically used for identification of the discriminatory markers and selection of the informative correlations between the markers and the modeled classes. Therefore, the filtered markers required certain conditions to be satisfied, specifically position in S plot $(|p| > 0.05 \text{ and } |p_{corr}| > 0.3)$, VIP value (>1.0) and *t*-test (p < 0.05) (Fig. 3E). As a result, the number of original variables was 5000. Then, 76 peaks were chosen as potential markers. Because the peak areas of most screened discriminatory variables were too low to obtain their MSⁿ data, a parent ion list-dynamic exclusion (PIL-DE)-based method for acquiring data was utilized to accomplish the comprehensive acquisition of HRMS1 and MSⁿ data sets, which greatly helped in the following structural identification.36

Markers 1, 2 and 3 yielded identical $[M-H]^-$ ions at an m/z value of 353.0867 (C₁₆H₂₃O₁₀, mass error within ±5 ppm) in

Table 3 Identification of discriminatory markers in SF and NSF FLJ using UHPLC-LTQ-Orbitrap MS

		Experimental						
No.	$t_{ m R}$	mass	Formula [M–H] [–]	MS/MS fragment ions	Identification			
M1 ^a	4.47	353.0869	C ₁₆ H ₁₇ O ₉	MS ² [353]: 191, 179, 135	3-CQA			
$M2^a$	6.91	353.0858	C ₁₆ H ₁₇ O ₉	MS ² [353]: 191, 179, 161	5-CQA			
$M3^{a}$	7.73	353.0856	C16H17O9	MS ² [353]: 173, 179, 191, 135	4-CQA			
$M4^{a}$	2.14	373.1122	C ₁₆ H ₂₁ O ₁₀	MS ² [373]: 193, 149, 167, 179, 119	Swertiamarin			
$M5^a$	5.30	373.1118	C ₁₆ H ₂₁ O ₁₀	MS ² [373]: 211, 167, 149, 193, 179	Secologanic acid			
M6	7.88	373.1118	C ₁₆ H ₂₁ O ₁₀	MS ² [373]: 193, 149, 167, 179	Swertiamarin isomer			
M7	4.23	375.1292	C ₁₆ H ₂₃ O ₁₀	MS ² [375]: 213, 169, 151	Loganin acid isomer			
M8	4.84	375.1280	C16H23O10	MS ² [375]: 213, 169, 151, 195	Loganin acid			
M9	5.84	375.1273	C ₁₆ H ₂₃ O ₁₀	MS ² [375]: 213, 169, 151	Loganin acid isomer			
M10	6.63	375.1292	C16H23O10	$MS^{2}[375]$: 195, 151	Loganin acid isomer			
M11	1.81	391.1231	C ₁₆ H ₂₃ O ₁₁	MS ² [391]: 229, 211, 193, 185, 167, 149	Secologanic acid hydrate			
M12	2.45	391.1255	C ₁₆ H ₂₃ O ₁₁	MS ² [391]: 211, 229, 193, 167, 149, 185	Secologanic acid hydrate			
M13	14.33	403.1223	C ₁₇ H ₂₃ O ₁₁	MS ² [403]: 371, 223, 179, 121, 91	Secologanin			
M14	1.63	433.0428	C ₁₆ H ₁₇ O ₁₂ S	MS ² [433]: 241, 415, 353, 161, 191, 287	CQA sulfate			
M15	2.53	433.0427	C ₁₆ H ₁₇ O ₁₂ S	MS ² [433]: 415, 387, 353, 241, 353	CQA sulfate			
M16	2.66	433.0433	C ₁₆ H ₁₇ O ₁₂ S	MS ² [433]: 241, 415, 387, 259, 353	CQA sulfate			
M17	4.62	433.0423	$C_{16}H_{17}O_{12}S$	$MS^{2}[433]: 415.387, 259$	CQA sulfate			
M18	5.01	433.0419	C ₁₆ H ₁₇ O ₁₂ S	MS ² [433]: 415, 241, 161, 259, 387	CQA sulfate			
M19	1.12	435.0591	$C_{16}H_{17}O_{12}S$	$MS^{2}[435]: 353, 191, 179$	CQA sulfite			
M20	3.15	437.0720	$C_{16}H_{21}O_{12}S$	$MS^{2}[437]: 193, 149, 373, 355$	Secologanic acid sulfite			
$M21^{a}$	19.06	447.0916	$C_{21}H_{19}O_{11}$	$MS^{2}[447]: 285$	Luteolin-7-0-glucoside			
M22	21.06	447.0918	$C_{21}H_{19}O_{11}$	$MS^{2}[447]: 285$	Luteolin-7-O-glucoside isomer			
M23	1.93	455.0822	C ₁₆ H ₂₃ O ₁₃ S	MS ² [455]: 373, 411, 437, 193, 211	Secologanic acid sulfite			
M24	2.15	455.0836	C ₁₆ H ₂₃ O ₁₃ S	MS ² [455]: 373, 437, 411, 193, 211	Secologanic acid sulfite			
M25	18.22	463.0854	$C_{21}H_{19}O_{12}$	$MS^{2}[463]: 301, 271, 445$	Hyperoside isomer			
M26	18.73	463.0861	$C_{21}H_{19}O_{12}$	$MS^{2}[463]: 301, 445, 271$	Hyperoside			
M27	23.05	499.1231	$C_{25}H_{23}O_{11}$	$MS^{2}[499]: 337, 173, 335, 353$	4- <i>p</i> Co-1-CQA			
M28	23.49	499.1233	C ₂₅ H ₂₃ O ₁₁	MS ² [499]: 353, 337, 191, 335, 179	5-pCo-3-CQA			
M29	25.21	499.1230	$C_{25}H_{23}O_{11}$	MS ² [499]: 353, 337, 179, 191	3-pCo-4-CQA			
$M30^{a}$	20.36	515.1155	$C_{25}H_{23}O_{11}$	MS ² [515]: 353, 335, 173, 179	3,4-DiCQA			
$M31^{a}$	20.85	515.1155	$C_{25}H_{23}O_{11}$	MS ² [515]: 353, 191, 179, 335	3,5-DiCQA			
$M32^a$	22.44	515.1163	$C_{25}H_{23}O_{11}$	MS ² [515]: 353, 191, 179, 335, 353	4,5-DiCQA			
M33	17.34	527.0494	$C_{21}H_{19}O_{14}S$	$MS^{2}[527]: 447, 285, 481$	Luteolin-7-O-glucoside sulfate			
M34	23.82	529.1343	$C_{26}H_{25}O_{12}$	MS ² [529]: 367, 179, 335, 353, 193	3-C-4-FQA			
M35	24.60	529.1340	C ₂₆ H ₂₅ O ₁₂	MS ² [529]: 353, 367, 191, 179	5-C-3-FQA			
M36	25.86	529.1335	C ₂₆ H ₂₅ O ₁₂	MS ² [529]: 353, 367, 173, 335	cis-5-C-3-FQA			
M37	8.73	543.0431	C21H19O15S	MS ² [543]: 463, 381, 525, 301	Hyperoside sulfate			
M38	12.76	543.0432	C21H19O15S	MS ² [543]: 381, 301, 381, 463	Hyperoside sulfate			
M39	18.80	593.1488	C ₂₇ H ₂₉ O ₁₅	$MS^{2}[593]: 285, 447$	Lonicerin isomer			
M40	19.71	593.1483	C27H29O15	MS ² [593]: 285, 447	Lonicerin isomer			
$M41^{a}$	20.50	593.1486	C ₂₇ H ₂₉ O ₁₅	$MS^{2}[593]: 285$	Lonicerin			
M42	16.70	595.0737	C ₂₅ H ₂₃ O ₁₅ S	MS ² [595]: 549, 577, 415, 241, 259	DiCQA sulfate			
M43	16.98	595.0750	C25H23O15S	MS ² [595]: 549, 577, 415, 301, 397	DiCQA sulfate			
M44	17.61	595.0748	C ₂₅ H ₂₃ O ₁₅ S	MS ² [595]: 577, 549, 415, 433, 241, 259	DiCQA sulfate			
M45	17.89	595.0737	C25H23O15S	MS ² [595]: 577, 549, 415, 433, 241, 259	DiCQA sulfate			
M46	19.38	595.0745	C25H23O15S	MS ² [595]: 577, 549, 415, 433, 259	DiCQA sulfate			
M47	21.25	595.0745	$C_{25}H_{23}O_{15}S$	MS ² [595]: 577, 415, 549, 433, 259, 241	DiCQA sulfate			
M48	22.70	607.1653	C28H31O15	MS ² [607]: 299	Chrysoeriol-7-0-β-D-neohesperidoside			
M49	18.30	609.1403	C27H29O16	MS ² [609]: 301, 300, 271, 255, 179, 591	Rutin			

^{*a*} Identified by comparison with reference standards; CQA, caffeoylquinic acid; DiCQA, dicaffeoylquinic acid; *p*CoCQA, *p*-coumaroylcaffeoylquinic acid; CFQA, caffeoylferuloylquinic acid.

negative ion mode. Their deprotonated molecular ions all generated a series of diagnostic fragment ions including those with m/z values of 191 [M–H–caffeoyl]⁻, 179 [caffeic–H]⁻ and 173 [M–H–caffeoyl–H₂O]⁻.³⁷ CQAs attributed to three different linkage positions of caffeoyl groups on quinic acid have been reported to display different intensities of their ESI-MS² base peak ions and predominant product ions. Meanwhile, based on

retention times and MS^n spectra of the corresponding reference substances and literature data, markers 1–3 were identified to be 5-CQA (Fig. S5†), 3-CQA and 4-CQA, respectively.

Markers 14–18 generated their deprotonated $[M-H]^$ molecular ions each at an m/z of 433.0435 ($C_{16}H_{17}O_{12}S$, mass error within ±5 ppm). In their ESI-MS² spectra, the diagnostic product ions were at m/z values of 415 $[M-H-H_2O]^-$, 387 [M-H-



Fig. 3 The mass fragmentation behaviors of identified markers. (A) HRMS¹ spectrum of M14. (B) ESI-MS² spectrum of M20. (C) HRMS¹ spectrum of M23. (D) ESI-MS² spectrum of M24. (E) The S-plot of LC-MS metabonomics analysis.

 $H_2O-CO]^-$, 353 [M-H-SO₃]⁻, 259 [caffeic-H + SO₃]⁻ and 241 [caffeic-H + SO₃-H₂O]⁻. The observation of the pair of ions at *m*/*z* values of 433 and 353 (Fig. 3A) further confirmed that the sulfate moiety was introduced to the CQA molecule, which has to the best of our knowledge never been reported before. Finally, markers 14–18 were tentatively identified as isomeric CQA sulfate.

Similarly, the ESI-MS² spectrum of marker 20 (Fig. 3B) showed an m/z signal corresponding to its deprotonated $[M-H]^-$ molecular ion at a value of 437.0748 ($C_{16}H_{21}O_{12}S$, error within ± 5 ppm). Moreover, the characteristic product ions at m/z values of 373 $[M-H-SO_2]^-$, 193 $[M-H-SO_2-Glc-H_2O]^-$ and 149 $[M-H-SO_2-Glc-H_2O-CO_2]^-$ were all observed. Based on the observation of the signals at the m/z values of 193 and 149 coupled with its $[M-H]^-$ ion, marker 20 may be concluded to be secologanic acid.³⁸ Meanwhile, the observation of the product ion at the m/z value of 373 confirmed that the sulfite moiety was introduced into the iridoid molecule. Accordingly, marker 20 was tentatively identified as isomeric secologanic acid sulfate.

In addition, a combination of the isotopic pattern combined and chromatography analyses was used for screening sulfurcontaining compounds in the complex systems, mainly because the ³⁴S isotopic ion has been shown to be drastically affected by ¹³C₂ and ¹⁸O.³⁹ Markers 23 and 24 produced their $[M-H]^-$ ions each at an *m*/*z* of 455.0822 (C₁₆H₂₃O₁₂S, error within ±5 ppm). And both of them generated a series of fragment ions at *m*/*z* values of 437 $[M-H-H_2O]^-$, 411 $[M-H-CO_2]^-$, 373 $[M-H-H_2SO_3]^-$, 211 $[M-H-H_2SO_3-Glc]^-$ and 193 $[M-H-H_2SO_3-Glc-H_2O]^-$. Furthermore, they simultaneously produced the isotopic patterns of the ³⁴S ion at an *m*/*z* of 457.07822 and of the ¹³C₂ + ¹⁸O ion at an m/z of 457.11760. Their characteristic product ions at m/z values of 437 and 373 probably resulted from the occurrence of the sulfite moiety in some of the iridoid molecules. Accordingly, markers 23 and 24 were putatively identified as secologanic acid-sulfite or its isomers (Fig. 3C and D).

Taken together, a total of 49 discriminatory markers (Table S4†) attributed to iridoids, organic acids and flavones were screened and characterized according to the fragmentation behaviors, isotopic patterns and diagnostic product ions obtained using the UHPLC-LTQ-Orbitrap MS coupled with the established integrated strategy. Eighteen of these markers were assigned to sulfate/sulfite derivatives of iridoid and chlorogenic acid, which could be chosen as the characteristic Q-markers for SF FLJ discrimination.⁴⁰ (Note that Fig. S6† shows a histogram of signal intensities of sulfur derivatives.)

3.3.4 Multi-omics correlation analysis (MOCA). At first, we performed a selection of specific wavenumbers according to the NIR-based metabonomics data analysis. Analysis of the available NIR spectra, specifically of the different NIR wavebands, quickly provided vast amounts of useful chemical information. However, it was in fact unable to present selective valid wavebands with discriminating potential. In order to interpret the sulfur fumigation process and screen the potential wavebands that presented the significant differences between SF and NSF samples, synergy interval partial least squares (siPLS) analysis with three intervals was employed to obtain the significant differences between SF and NSF samples. To eliminate the influence of overfitting, we set the latent variables to be within the range 1–10. As demonstrated in Table 4, the siPLS analysis with SG11+2nd optimized (the other siPLS methods are shown

	PCA			PLS-DA			
Preprocessing method	Lv	$R^2(X)$	$R^2(Y)$	Lvs	$R^2(X)$	$R^2(Y)$	Q^2
Baseline	3	0.998	0.997	3	0.997	0.501	0.326
Spectroscopic transformation	3	0.999	0.998	3	0.999	0.390	0.248
MSC	6	0.999	0.997	3	0.858	0.454	0.206
Normalization	5	0.999	0.999	3	0.975	0.494	0.309
Original	3	0.999	0.999	3	0.999	0.497	0.249
SG91 st	5	0.899	0.831	4	0.845	0.827	0.601
SG92 nd	5	0.621	0.381	3	0.425	0.827	0.309
SG111 st	4	0.693	0.578	3	0.583	0.791	0.476
SG112 nd	6	0.582	0.234	3	0.292	0.922	0.418
SNV	4	0.997	0.996	3	0.993	0.517	0.336
WDS	3	0.999	0.999	3	0.999	0.538	0.159

Table 4The results of SiPLS analysis

in Fig. S7 and S8†) was the best discriminatory model and achieved a value of 0.859 for $R^2(Y)$ and 0.471 for Q^2 ; *i.e.*, this method produced a better model performance than did any of the other preprocessing methods. Furthermore, the SF and NSF FLJ samples were clearly separated (Fig. 4A). The wavenumbers of the optical subinterval combinations ranged from 5000 to 5200 cm⁻¹. Thereafter, NIR spectra were integrated with two-dimensional correlation spectra (2D-COS) that were expected to clearly identify the screened wavebands, discriminate features and details of the structural changes of SF and NSF FLJ samples. For the synchronous 2D-COS auto-peak analysis, two main sensitive variables were identified and displayed wavenumbers ranging from 5000 to 5200 cm⁻¹ (see Fig. 4B, which

shows the diagonal data of the 2D-COS plot, and Fig. S9,† which shows the 2D-COS plot), whose correlation analysis was in accordance with the wavebands screened using the siPLS method. Similar to that previously reported, strong absorptions in the wavenumber range $5000-5200 \text{ cm}^{-1}$ were attributed to S–H and S–OH.^{33,41} As mentioned above, some sulfur-containing components were identified as the discriminatory markers. As a result, a close relationship was identified between the screened wavebands ($5000-5200 \text{ cm}^{-1}$) and the sulfurcontaining markers generated in the sulfur fumigation process.

To validate the above-mentioned results, one of the main representative chemical constituents, namely chlorogenic acid (5-CQA), was subjected to sulfur fumigation and analyzed using



Fig. 4 The MOCA for the FLJ. (A) The discriminatory information of preprocess method of SG9+1st; (B) the synchronous 2D-COS auto-peak analysis of the SF and NSF samples; (C) the mass fragmentation behaviors of SF chlorogenic acid; (D) the synchronous 2D-COS auto-peak analysis of the SF and NSF chlorogenic acid samples; (E) the mass fragmentation behaviors of SF chlorogenic acid.

the same methods. The autocorrelation curves of the SF and NSF chlorogenic acid samples (Fig. 4D) were derived from their respective 2D-COS spectra. Obvious differences between the SF and NSF chlorogenic acid samples in the wavebands between 5000 and 5200 $\rm cm^{-1}$ were observed, which was also in accordance with the wavebands screened using the siPLS model. The subsequent LC-HRMS analysis of an SF chlorogenic acid mixture also indicated the presence of newly generated constituents (Fig. 4C and E) except for the prototype drug during the process of sulfur fumigation. Through analyzing the fragment ions of the S-derivatives, it was found that SO₃ (79.9568) and H₂SO₃ (81.9725) were the characteristic neutral losses of organic sulfates or sulfites. The assignments of these newly emerged peaks were confirmed to be the rudimentary sulfate derivatives of chlorogenic acid based on the HRMS data, which indicated a mass 79.95 Da (SO₃) more than that of standard reference. It also indicated that the results of NIR were reliable and credible in the discrimination of SF FLI.

3.4 Proposed protocol for the rapid detection and mechanistic explanation of the sulfur fumigation of CHMs using SF FLJ as a study case

Evaluating the quality of CHMs and judging their authenticity are two major challenges. Sulfur fumigation has attracted increasing attention due to its alteration of CHM quality resulting from its damage to bioactive components, generating excessive sulfur dioxide residue and especially changing the chemically active ingredients in CHMs.

NIR, HPLC and LC-MS were proposed to be used to evaluate the quality of CHMs. However, the variations during sulfur fumigation were much more complicated than expected. Furthermore, the amount of data obtained based on one single method was still limited, making it difficult to expound on the mechanism of sulfur fumigation. To experimentally support our inference, we selected FLJ as a model herb in this study. With the development of a few high-throughput strategies, integrative metabonomics analysis was applied to integrate the multiple interactions of NIR spectra, HPLC chromatograms and HRMS data. The results aimed to reveal whether the herb underwent sulfur fumigation and to illuminate the inherent mechanism of the NIR judgment method by associating NIR with HPLC and UHPLC-MS analyses. Our established approach was applied to rapidly discriminate SF FLJ among many unknown samples, and is expected to be greatly beneficial for guaranteeing CHM quality.

To perform the analysis of the sulfur fumigation of CHMs, the process of sulfur fumigation was first simulated in the laboratory. According to the characteristics of each analytical technology, optimum analytical conditions were adopted and the corresponding high-quality data of SF and NSF CHM samples were



Fig. 5 A suggested protocol for the rapid discrimination of SF CHMs and an explanatory mechanism.

obtained. All of these experiments provided the foundation for subsequent data analysis, which is illustrated in Fig. 5.

Step 1: Performing PCA and PLS-DA for the single technology.

This step was focused on the analytical capability of each single technology and whether the SF and NSF CHMs could be distinguished. Our study demonstrated that NIR spectroscopy based on a data preprocessing method (SG9+2nd) with a multivariate calibration approach such as PCA and PLS-DA was the appropriate tool to discriminate SF from NSF FLJ samples. The chemical constituents in FLJ samples displayed strong ultraviolet absorption, which was observed with HPLC-DAD at 330 nm, 238 nm, 254 nm and 280 nm. Peak areas (≥150 mAU) were selected separately through the data fusion of the four wavelengths and then analyzed by performing PCA and PLS-DA. In addition, a UHPLC-LTQ-Orbitrap high-resolution MS was employed to comprehensively and dynamically profile the chemical constituents in FLJ. The derivative content during the sulfur fumigation process was not abundant enough, *i.e.*, the signals of sulfur-containing analytes would have been drowned out by the contribution of inherent constituents.

Step 2 and Step 3: Performing the integrative metabonomics analysis, such as mid-level metabonomics data fusion analysis without/with variable selection.

In our previous study, the data fusion of NIR- and HRMSbased metabonomics-like analysis was successfully applied to accomplishing the discrimination of SF Ophiopogon Radix.¹⁷ Herein, we combined three kinds of analytical techniques including NIR, HPLC and UHPLC-HRMS to obtain the dimensional information of SF samples, and investigated two types of mid-level metabonomics data fusion strategies as illustrated in Step 2 and Step 3. For that, informative features of the raw data from a single instrument were separately extracted using their own protocol from sample preparation to data preprocessing.

The comparison between the unique model and the metabonomics data fusion model is illustrated in Fig. 6. No single analytical platform could be utilized to accurately discriminate the SF samples based on PCA score plots. HPLC and NIR led to classification without rhyme or reason and HRMS could not correctly discriminate one of the SF samples (no. 16). Thus, we believed that utilizing mid-level metabonomics data fusion without variable selection to obtain more accurate characteristics of the samples might be a much better choice. As a result, the potential to discriminate between of NSF and SF samples was actually improved with no. 16 still in the wrong class, and the results were worse than the individual application of MS. Mid-level fusion with variable selection was employed and clearly improved the class separation, as samples were correctly classified and less scattered (Fig. 6K). Taking the classification into account, the fusion of NIR and HRMS data, accomplished with high accuracy, provided the best model (Fig. 6I and J). Moreover, variables that were selected before classification generated better classification results than those obtained when all variables were used. Overall, the proposed metabonomics data fusion approach demonstrated an ability to effectively discriminate key information from raw analysis data.



Fig. 6 Comparison between the unique model and the metabonomics data fusion model. (A–C) PCA for HPLC-DAD, NIR and LC-MS. (D–G) Result for HPLC-NIR, HPLC-MS, NIR-MS and NIR-HPLC-MS data fusion without variable selection analysis. (H–K) Result for HPLC-NIR, HPLC-MS, NIR-MS and NIR-HPLC-MS data fusion with variable selection analysis.

The results demonstrated that the mid-level metabonomics data fusion methods were much better than all of the primary analyses, which meant that the information obtained from individual techniques was in fact insufficient. The results from both kinds of mid-level fusion strategies accomplished the effective discrimination of SF FLJ samples.

Step 4: Identifying the discriminatory markers attributed to group separation.

LTQ-Orbitrap high-resolution MS has been one of the most powerful approaches used for the rapid identification of multiple constituents in CHMs.^{42,43} It has been used to combine high trapping capacity and multiple data acquisition of linear ion traps to generate a large amount of information from MS^1 and MS^n data. In this study, a highly sensitive and effective strategy was utilized for rapidly screening and identifying SF FLJ by using PIL-DE acquisition based on a hybrid LTQ-Orbitrap mass spectrometer to accomplish the overall acquisition of data sets, which helped allow for a search of a greater number of potential active compounds especially for the sulfur-containing constituents. As a result, 49 markers including iridoids, organic acids and the sulfur-containing derivatives were positively or tentatively identified.

Step 5: Application of MOCA for deriving mechanistic explanations of the sulfur fumigation process and the corresponding method validation.

An NIR spectrum was constructed from different wavebands, but not every waveband displayed a special discrimination ability. Therefore, siPLS analysis was employed to screen the potential wavebands that presented the significant differences between SF and NSF samples. In step 4 mentioned above, some sulfur-containing constituents were identified that would explain the potential NIR wavebands. Chlorogenic acid was selected as the example to validate whether the new sulfurcontaining derivatives were produced after the sulfur fumigation process.

4. Conclusions

Our work indicated that the proposed protocol for the rapid detection of SF CHMs is also beneficial for revealing the intrinsic mechanism of sulfur fumigation and boosting the ability to discriminate SF from NSF FLJ samples, and could serve as an example for future research on rapidly detecting other SF CHMs. Integrative metabonomics analysis was also found to be beneficial for evaluating the quality of and rapidly detecting CHMs. Our work also suggests a future trend of integrating multiple metabonomics datasets from different technologies to achieve a sound evaluation.

Conflicts of interest

All authors declare that they have no conflict of interest.

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