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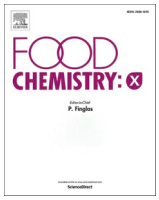

Quality evaluation and identification of *Houttuynia cordata* bleached with sodium metabisulfite based on whole spectrum metabolomics

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ABSTRACT

Houttuynia Cordata (HC) is a widely distributed plant in Asia and is used extensively for both food and medicinal purposes. A preliminary investigation found that HC is often bleached with sodium metabisulfite solution during its field processing, leading to health risks. In this study, the effects of sodium metabisulfite on the quality of HC were comprehensively evaluated using volatile and non-volatile targeted metabolomic methods. The results revealed a positive correlation between the extent of chemical composition changes and the bleaching time. These notable changes mainly occurred at the initial stage of bleaching. Subsequently, an untargeted UPLC/Q-TOF MS method was used to explore the potential chemical bleaching markers in bleached HC. The marker 1-hydroxy-3-oxodecane-1-sulfonic acid was subsequently prepared, isolated, and identified. Market sample verification further validated the accuracy and effectiveness of this marker.

1. Introduction

Houttuynia cordata (HC, Yu-xing-cao in Chinese) is a widely distributed plant in East, South, and Southeast Asia (Fu, Dai, Lin, & Lu, 2013). It is used as both traditional food and medicinal materials (Kumar, Prasad, & Hemalatha, 2014). In India, HC is used for various ethnomedical purposes in many forms, such as medicinal salad, leaf juice, and pot-herb. In Southwest China, mainly in the Guizhou, Sichuan, and Yunnan provinces, the fresh-cut root of HC, commonly known in Chinese as Zhe-er-gen, is a specialty condiment among locals. When used for medical purposes, the whole fresh plant or dried aerial part is traditionally used for clearing heat, eliminating toxins, reducing swelling, and relieving stagnation (Kumar et al., 2014; Shingnaisui, Dey, Manna, & Kalita, 2018). Modern pharmaceutical studies have shown that its main components are volatile oil, flavonoids, and alkaloids (Wu et al., 2022; Ju et al., 2021). Currently, HC is also used as a common raw material in the production of various modern injections and Chinese patent medicines (Wu, Ding, Liu, Dai, & Ma, 2022).

Our preliminary investigation found a “bleaching” phenomenon in processing HC raw materials. The clean and fresh HC plants are soaked in a high concentration of sodium metabisulfite solution ($Na_2S_2O_5$) to achieve a whitening effect. The bleached HC has the characteristics of being whiter in color, crisp and tender in taste, less intense in odor, and an extended shelf-life product. This bleaching phenomenon is gaining popularity and is challenging to control due to its low cost and simple operation.

Sulfite food additives, mainly used as SO_2 release agents, are frequently employed in the postharvest processing of various agricultural products for flavoring, packing, and enhancing food appearance (Martínez-Blay, Taberner, Pérez-Gago, & Palou, 2021). Sodium metabisulfite is an extensively used sulfite (Ahmadi, Lee, Lee, Oh, Park, & Kwak, 2018). It has been widely used in various foods, such as potatos (Nascimento, Canteri, Rodrigues, & Kovaleski, 2020), pepper (Bao et al., 2022), fungus (Ren et al., 2022), and various fruits (Ahmadi et al., 2018; Krongchai, Jakmunee & Kittiwachana, 2020). However, recently, the toxicological effects (Alimohammadi et al., 2021; Ghasemi, Salari, Kalantarmahdavi, & Amiryousefi, 2022) induced by sulfate agents including sodium metabisulfite in human organs like the lungs (Vally & Misso, 2012) and nervous system (Lai et al., 2018), and the allergic reactions (Borges, Valejo Coelho, Fernandes, Brasileiro, & Gonçalo,

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2018) have gradually attracted attention. In addition to the direct safety risks resulting from the high residue of sodium metabisulfite, there may be potential risks associated with the alteration in the internal quality of products that undergo sulfite processing. Previous studies have demonstrated that sodium metabisulfite can significantly alter the chemical composition and content of pepper (Bao et al., 2022) and black fungus (Ren et al., 2022). Furthermore, unlike studies on common foods that primarily focus on primary metabolites or flavor substances, research into dual-purpose medicinal and edible products like HC needs to explore further the changes of special metabolites, the key pharmacologically active components, caused by bleaching. In similar instances of sulfur fumigation, sulfur fumigation has been reported to cause significant quality changes in many medicinal plants like gastrodia elata (Kang et al., 2017), moutan cortex (Zhan et al., 2018), and ginseng (Zhang et al., 2021). However, the potential changes in specific metabolites caused by sulfites like sodium metabisulfite are rarely reported. Furthermore, from a product regulatory perspective, in identifying products bleached with sulfate agents, the discovery of specific chemical identification markers with the sulfur-containing compounds generated during the bleaching process has been gradually developed alongside traditional SO_2 residue analysis (Kang et al., 2017).

In this study, the effects of bleaching on the quality of HC with sodium metabisulfite were first investigated. We aimed to evaluate the appearance characteristics and sulfur dioxide residues and employed both volatile and non-volatile targeted metabolomic methods to detect changes in chemical composition. Subsequently, we conducted a screening for potential sulfur-containing markers that could rapidly identify bleached HC samples using a metabolomics-based UPLC/Q-TOF MS method. Lastly, the marker's preparation, separation, structural identification, and validation verification were performed.

2. Materials and methods

2.1. Chemicals, reagents, and herbal materials

Deionized water was purified using a Pacific T-II system (Thermo, USA), formic acid (HPLC grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA), methanol and acetonitrile (HPLC grade) were sourced from Merck Co. (Darmstadt, GER), tetrabutylammonium hydroxide (Lot *NO.* T1733364B) was obtained from Acmec Biochemical Technology (Shanghai, China). All other reagents used in this study were of analytical grade.

Fresh HC plant samples were collected from Lingchuan County, Guangxi Province in August 2023 for laboratory processing. A total of 15 batches of commercial HC samples were collected from the market for the validation of the bleaching marker obtained in the study (Table S1). All samples were identified by Prof. Weike Jiang from Guizhou University of Traditional Chinese Medicine as *Houttuynia cordata*. The voucher specimens were deposited in the National Resource Center for Chinese Materia Medica, China Academy of Chinese Medical Sciences (Beijing).

2.2. Bleaching of HC samples with sodium metabisulfite

Considering the actual production situation, the underground parts of fresh HC plants were treated with a sodium metabisulfite solution at 20 g/L. For further investigation on the influence of soaking time on HC, five treatment levels were set as 0 h (T-0), 1 h (T-1), 2 h (T-2), 4 h (T-) and 8 h (T-8). The specific operation was conducted as follows: 5 kg plant samples were washed and spread out to dry off surface water. The samples were then divided randomly and evenly into five groups and placed into separate plastic boxes. Each box was filled with 10 L of sodium metabisulfite solution to submerge the samples and treated for a specific duration. The samples were then freeze-dried, ground into powder (Retsch MM400, Haan, Germany), and passed through a 50-mesh sieve. Lastly, the dried *Houttuynia cordata* powder (DHP) samples were sealed in polythene bags and stored at –20 °C for further analysis. All samples were prepared in triplicate.

2.3. Sulfur dioxide residue analysis

The sulfur dioxide residue of HC samples was determined by the iodine titration method according to the *Chinese Pharmacopoeia 2020 version* (Part Four) Appendix 2331.

2.4. Influence of bleaching on both non-volatile and volatile chemical constituents of HC based on targeted metabolomics analysis

2.4.1. Sample preparation for UPLC-MS/MS

A total of 50 mg of DHP sample was weighed, and 1200 μL of a 70 % methanol extract, pre-cooled to –20 °C and containing internal standard, was added. The mixture was then vortexed once every 30 min for 30 s for six times. After centrifugation at 12000 rpm for 3 min, the supernatant was aspirated and filtered through a microporous membrane (0.22 μm) to prepare the test sample.

2.4.2. UPLC-MS/MS conditions

The test samples were analyzed using a UPLC-ESI-MS/MS system (UPLC, ExionLC™ AD, MS, Applied Biosystems 6500 + Q TRAP). The analytical conditions were as follows: for UPLC, the column was Agilent SB-C18 (1.8 μm, 2.1 mm × 100 mm). The mobile phase consisted of pure water with 0.1 % formic acid (solvent A) and acetonitrile with 0.1 % formic acid (solvent B). The gradient elution program was (0–9.0) min, 5 %→95 % B; (9.0–10.0) min, 95 %→95 % B; (10.0–11.1) min, 95 %→5% B; (11.1–14.0) min, 5 %→5% B. The flow rate was 0.35 mL/min, the column temperature was 40 °C, and the injection volume was 4 μL.

The ESI-MS parameters were as follows: source temperature was 550 °C, ion spray voltage (IS) was 5500 V in positive ion mode, and –4500 V in negative. Ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 50, 60, and 25 psi, respectively. The QQQ scans were acquired in MRM mode. The declustering potential and collision energy for individual MRM transitions were further optimized. A specific set of MRM transitions was monitored for each period based on the metabolites eluted within this period.

2.4.3. Sample preparation for GC-MS

A total of 500 mg of DHP sample was transferred to a 20 mL headspace vial and sealed using crimp-top caps with TFE-silicone headspace septa. For SPME analysis, the vial was heated to 60 °C for 5 min. Subsequently, a 120 μm DVB/CWR/PDMS fiber was exposed to sample's headspace for 15 min at 100 °C.

2.4.4. GC-MS analysis conditions

The identification and quantification of volatile components were conducted using an Agilent Model 8890 GC and a 7000D mass spectrometer (Agilent) equipped with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm). Volatile components were desorbed from the fiber coating at 250 °C for 5 min in the splitless mode. Helium was used as the carrier gas at a linear velocity of 1.2 mL/min. The injector temperature was kept at 250 °C. The temperature program for the oven started at 40 °C, maintained for 3.5 min, then ramped up at a rate of 10 °C/min to 100 °C, followed by an increase of 7 °C/min to 180 °C, then accelerated at 25 °C/min to 280 °C, where it was held for 5 min. The MS was in EI ionization mode at 70 eV, quadrupole mass detector at 150 °C, ion source at 230 °C, and transfer line temperatures at 280 °C. The identification and quantification of analytes were in ion monitoring (SIM) mode.

2.4.5. Multivariate statistical analysis

Data obtained from UPLC-ESI-QTRAP-MS/MS was analyzed using Analyst (version 1.6.3) and Multiquant (version 3.0.3) software. Data obtained from GC-MS was analyzed using Agilent MassHunter

Workstation software. Metabolites were tentatively identified by comparison with MS/MS spectrum information from published databases and standards from the MetWare metabolite database. Next, the two data sets were exported and merged in Excel to facilitate subsequent multivariate statistical analysis. The unsupervised principal component analysis (PCA), heatmap analysis, and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed by the R package MetaboAnalystR. The data was log transformed (log2) and mean-centered before analysis, and a permutation test (200 permutations) was performed during OPLS-DA. The differential metabolites were determined by parameters $p < 0.05$, VIP > 1, and fold-change $|Log_2FC| \geq 1.0$.

2.5. UPLC/Q-TOF MS guided screening and identification of bleaching markers in HC

2.5.1. Sample preparation

A total of 0.3 g DHP sample was weighed precisely and added with 1.5 mL 70 % (v/v) methanol. Then this sample was extracted by ultrasonication for 30 min at 40 kHz (SCIENT ultrasonic processor, Ningbo, China). After cooling, the extract was centrifuged for 10 min at 12,000 g and the supernatant was filtered through a 0.22 μm syringe filter as the test sample. A mixture of all sample solutions was used as the control sample (QC) and was analyzed at the start and after every two samples for quality control analyses.

2.5.2. UPLC/Q-TOF MS conditions

The separation was performed using a UPLC system (Shimadzu Corp., Japan) with an ACQUITY UPLC HSS T3 column (1.7 μm, 2.1 mm × 100 mm). The flow rate was 0.5 mL/min and the column temperature was 30 °C. The mobile phase was 0.1 % formic acid aqueous solution (A) and acetonitrile (B). The gradient elution program was as follows: (0–6.0) min, 5 %→16 % B; (6.0–9.0) min, 16 %→16 % B; (9.0–15.0) min, 16 %→47 % B; (15.0–20.0) min, 47 %→55 % B; (20.0–24.0) min, 55 %→78 % B; (24.0–25.0) min, 78 %→95 % B; (25.0–28.0) min, 95 %→95 % B. The injection volume was 1 μL.

The MS was performed using an Applied Biosystems SCIEX X500R QTOF system. The data acquisition mode was IDA and the experiment was performed in ESI negative ionization mode. The parameters were set as follows: Ion source gas (GS1) was 50 psi, Ion source gas II (GS2) was 50 psi, Curtain gas (CUR) was 35 psi, and CAD gas (CAD) was 7 psi. Temperature (TEM) was 550 °C. For MS1, the acquisition range was 100 ~ 1000 Da, DP was –80 V, CE was –10 V, and accumulation time was 0.25 s. For MS2, the acquisition range was 50 ~ 1000 Da, DP was –80 V, CE was –35 V, and mass tolerance was 50 mDa. The accumulation time was set at 0.04 s. The 10 most abundant ions per cycle were selected for MS/MS and dynamic exclusion was used with an exclusion duration of 6 s.

2.5.3. Data processing and multivariate analysis

The raw data obtained from UPLC/Q-TOF MS was converted with AnalysisBaseFileConverter v4.0.0 software and processed in MSDIAL v5.1. The adduct ions selected were $[M\text{-}H]^-$, $[M + FA]^-$, and $[M + Cl]^-$, with a peak extraction time ranging from 0–28 min. After peak detection and alignment, the data was exported to MetaboAnalyst v5.0 (https://www.metaboanalyst.ca) for PCA and OPLS-DA. Data with more than 20 % missing values were removed. The remaining missing values were replaced with half the minimum positive value in the original data. The data was Pareto-scaled before PCA and OPLS-DA.

Both public databases including MassBank, LipidBlast, and GNPS, and an in-house library were employed to characterize the metabolites. After being matched with the database information, the candidate compounds were manually checked according to their mass fragmentation mechanism and retention time. A Neutral Loss method (Wu et al., 2022), including the characteristic mass loss $\triangle m/z = 81.97$ Da caused by the loss of an H_2SO_3 and $\triangle m/z = 79.95$ Da produced by the loss of an SO_3, was used to rapidly identify the characteristic sulfonated markers produced by the bleaching with sodium metabisulfite.

2.5.4. Isolation, purification, and identification of chemical bleaching marker

Fresh HC samples were sectioned and soaked in 20 % sodium bisulfite aqueous solution of 5 times the quantity, stirred thoroughly, and stayed at room temperature for 72 h. Then, the filtered solution was extracted twice with *n*-butanol and the *n*-butanol layers were combined and concentrated with an EYELA N-3010 rotary evaporator (Tokyo Rikakikai, Ltd., Tokyo, Japan) at 60 °C. Next, the residue was dissolved in water, and the aqueous phase was extracted twice using ethyl acetate. The ethyl acetate layers were concentrated to nearly dry and the residue was dissolved in a mixture of water and DMSO reagent. The solution was filtered and then prepared and purified using a Waters 2555–2489 high-performance preparative liquid chromatography system with the following parameters: mobile phase was acetonitrile (70 %) and 0.03 % tetrabutylammonium hydroxide aqueous solution (30 %), the flow rate was 100 mL/min, detection wavelength was 280 nm. The fraction above 95.0 % was collected and concentrated with a rotary evaporator at 60 °C to remove the organic phase. The remaining aqueous phase was extracted twice with ethyl acetate. The ethyl acetate phase was then washed twice with a saturated NaCl aqueous solution. Finally, the ethyl acetate phase was concentrated under reduced pressure at 30 °C, and the product marker Peak 23 was obtained as a pale-yellow oil. The product was dissolved in DMSO-d_6 and identified by NMR (Agilent 400MRI).

2.6. Quantitative analysis of chemical bleaching marker

2.6.1. Sample preparation

A total of 0.2 g DHP sample was weighed precisely and added with 25 mL 70 % (v/v) methanol. The sample was then extracted by ultrasonication for 30 min at 40 kHz. After cooling, the extract was filtered. Subsequently, the filtrate was filtered through a 0.22 μm syringe filter as the test sample.

2.6.2. UPLC-UV conditions

The separation was performed using an Agilent 1290 II UPLC system with an ACQUITY UPLC HSS T3 column (1.7 μm, 2.1 mm × 100 mm). The mobile phase was methanol and 10 % tetrabutyl ammonium hydroxide aqueous solution (70: 30). The flow rate was 0.2 mL/min and the column temperature was 30 °C. The injection volume was 2 μL. The detection wavelength was set at 283 nm.

2.6.3. Method validation

The quantitative analysis method of marker 23 was validated by using analytical parameters including specificity, linearity, the limit of detection (LOD), the limit of quantitation (LOQ), precision, accuracy, and stability.

3. Results and discussion

3.1. Effects of bleaching treatment on the appearance characters of HC

In terms of appearance and characteristics (Fig. 1A), there was no noticeable difference in appearance among the HC samples in a short time after bleaching. However, the T-4 and T-8 samples, which were soaked for an extended period, developed a strong, pungent sulfide odor. The bleaching effect gradually appeared with the increase in storage time. Compared to the T-0 group, the bleached samples exhibited a whiter color and crisper texture and were less prone to dehydration, indicating an extended harder to get dehydrated indicating an extended storage life. The T-0 samples began to visibly wither and discolor by 48 h post-bleaching, while the bleached samples remained fresh. At 72 h post-bleaching, the dehydration and color change became more evident in T-0 samples. However, these characteristics only appeared in a few

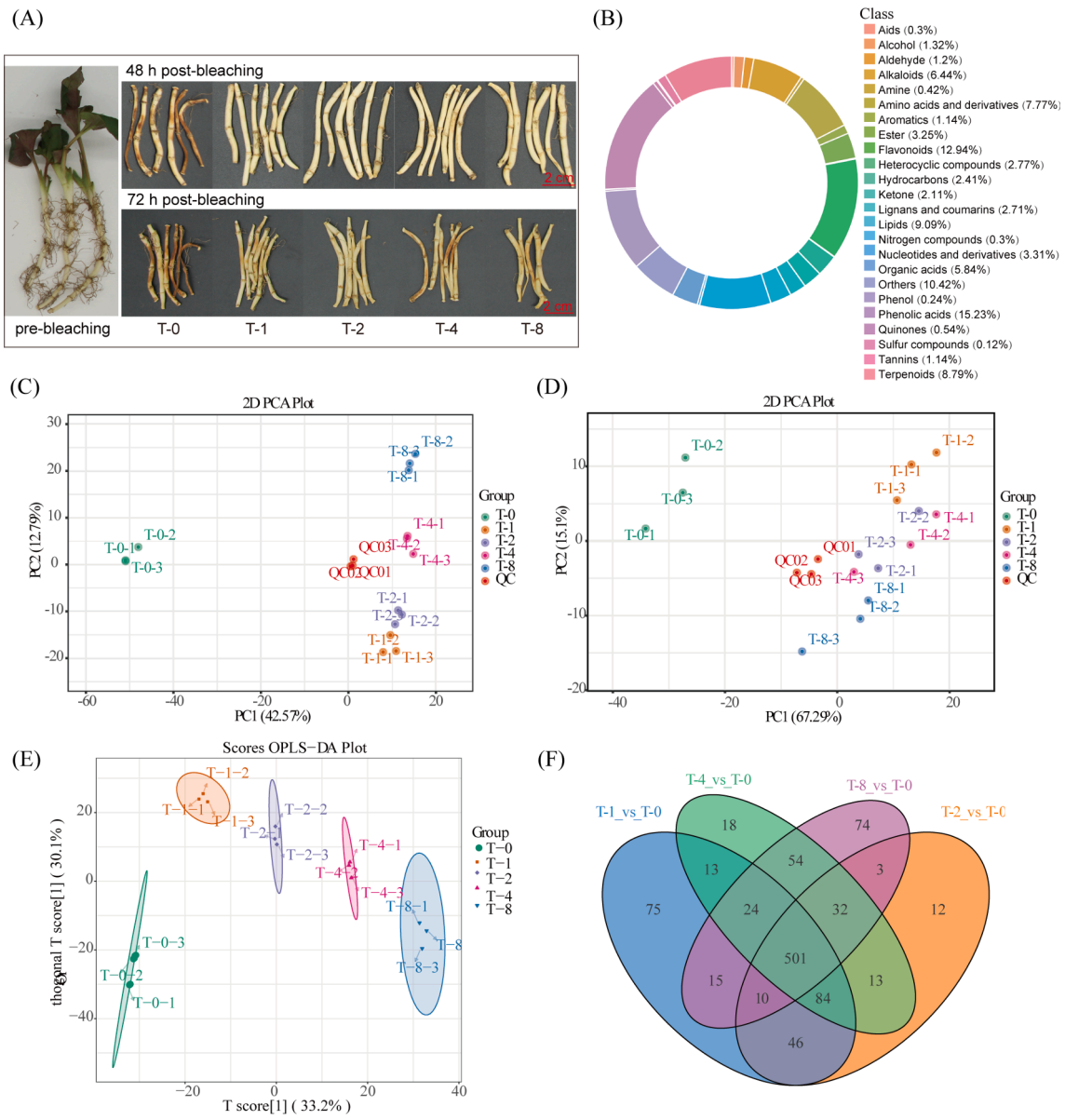


Fig. 1. Effects of bleaching on the quality of HC samples with sodium metabisulfite. (A) appearance characters of HC before and after bleaching. (B) Classification and composition of all metabolites detected in HC samples. (C) PCA score plot based on LC-MS data. (D) PCA score plot based on GC-MS data. (E) OPLS-DA score plot based on merged data of LC-MS and GC-MS. (F) Venn diagram of differential metabolites between T-0 and other groups.

samples of bleached groups. Interestingly, samples T-4 and T-8 showed more deterioration compared to the T-1 and T-2 groups. This indicates that sodium metabisulfite's bleaching and preservative effects on HC are not positively correlated with treatment duration. Further research is needed to understand the specific causes of this phenomenon.

The sulfur dioxide residue analysis of HC samples (Fig. S1) showed a positive correlation between sulfur dioxide residue in HC and the bleaching time. The sulfur dioxide residue reached 337 mg/kg in HC after bleaching for 1 h. The residue was higher than 900 mg/kg after bleaching for 8 h, which all exceeded the relevant limits and indicated a potential health risk.

3.2. Effects of bleaching on chemical constituents of HC based on targeted metabolomics analysis

3.2.1. Overall determination of non-volatile and volatile metabolites in HC samples

The analysis of QC samples showed (Fig. S2A–C) a high overlap ratio of the total ion current chromatogram curves. The data dispersion analysis showed that the proportion of chemical components with CV values less than 0.3 % in QC samples was 85 % by LC-MS and 100 % by GC–MS (Fig. S2D–E). From these results, it can be implied that the method and data were repeatable, reliable, and stable.

Lastly, a total of 1294 non-volatile metabolites and 367 volatile metabolites were tentatively identified in HC samples (Fig. 1B), mainly including 253 phenolic acids, 215 flavonoids, 151 lipids, 146 terpenoids, 129 amino acids and derivatives, 107 alkaloids, 97 organic acids, 55 nucleotides and derivatives, 54 ester, and 173 other metabolites. Among them, the first five kinds of metabolites accounted for more than 50 % and were regarded as the main compounds in HC.

Both the PCA analysis based on non-volatile components (Fig. 1C) and volatile components (Fig. 1D) displayed that the bleaching caused significant changes in the chemical composition of HC. The score plots show that bleached and unbleached samples can be divided into two groups respectively. Then, an OPLS-DA ($R^2Y = 0.999$, $Q^2 = 0.963$) was operated with the merge data of LC-MS and GC–MS. The score plots

(Fig. 1E) showed a continuous distribution trend between samples bleached with sodium metabisulfite from 0 to 8 h, which indicated that the change of chemical components in HC caused by the bleaching tends to be a continuous changing process over the treatment duration.

The variation of components in different sample groups was further analyzed. Metabolites with VIP > 1 and $p < 0.05$ were selected as differential metabolites and are presented in Table S2. The numbers of differential metabolites between T-0 and T-1, T-2, T-4, and T-8 groups were 768 (347 down and 421 up), 701 (306 down and 395 up), 739 (368 down and 371 up), and 713 (438 down and 275 up), respectively. Venn diagram (Fig. 1F) demonstrated 501 common components (Table S3) in the differential components between each bleaching group and T-0, accounting for most different metabolites between each group. This result suggests that significant alterations in the metabolites of HC caused by bleaching occur predominantly in the early stages. With the increase in treatment time, further differences mainly manifested as the changes in the relative contents of some common metabolites. This is consistent with the continuous distribution of the samples in the OPLS-DA score plot. The K-means clustering analysis based on the 501 differential metabolites (Fig. S3) showed that these metabolites were

Fig. 2. Analysis of differential metabolites between T-0 and T-1 samples. (A)OPLS-DA score plots. (B) Volcano plots. (C) Heatmap and proportions of differential metabolites between T-0 and T-1 groups. (D) Each of the top 10 differential metabolites screened from increased and decreased metabolites. (E) Aroma profiles based on differential metabolites data.

divided into 9 classes, and significant changes occurred in T-1 in most instances. Statistically, the content of about 51.5 % of metabolites (subclass 1 and subclass 2) declined significantly after 1 h of bleaching. These were mainly non-volatile metabolites including phenolic acids, lipids, amino acids and their derivatives, and flavonoids. Conversely, the rest of the metabolites (subclasses 3–8) showed an increasing trend after the bleaching and were mainly volatile metabolites consisting of hydrocarbons, terpenoids, and heterocyclic compounds. Some phenolic acids and flavonoids in subclass 9 increased within 1 h of bleaching but then declined following bleaching.

3.2.2. *Exploration of major differential metabolites in HC caused by bleaching*

The differential metabolites between T-0 and T-1 samples were further analysed based on previous analysis. The score plots of OPLS-DA (Fig. 2A) displayed a distinct clustering of two kinds of samples. As volcano plots showed (Fig. 2B), 421 components increased and 347 components decreased in HC after bleached with sodium metabisulfite.

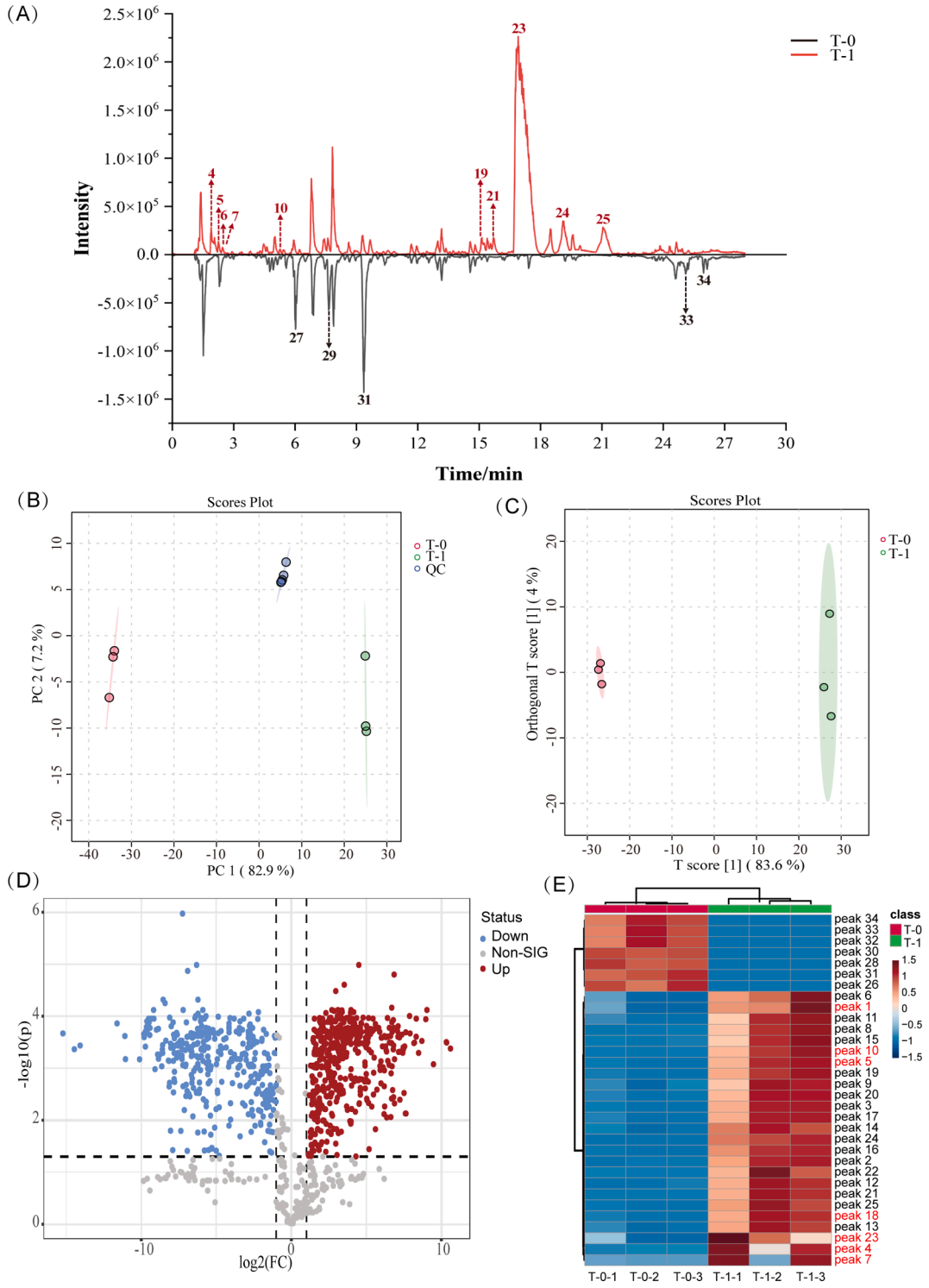


Fig. 3. Screening and identification of chemical bleaching markers of HC. (A) The base peak chromatograms of bleached (T-1) and un-bleached (T-0) HC samples. (B) PCA score plots. (C) OPLS-DA score plots. (D) Volcano plots. (E) Heatmap of 34 differential metabolites.

The heatmap (Fig. 2C) showed the overall distribution of the differential metabolites between T-0 and T-1 samples. Finally, according to the log_2FC value, each top 10 differential metabolite was screened from the increased and decreased metabolites, respectively, as displayed in Fig. 2D and Table S4. Results revealed that the 10 metabolites whose contents increased were mainly phenolic acids, flavonoids, and volatile oils (ketones and terpenes), and the 10 decreased were mainly lipid components.

To characterize the odor changes caused by bleaching, 73 metabolites with relative odor activity values (rOAV) larger than one (Yue et al., 2023) were screened out from the differential volatile metabolites detected by GC-MS. The aroma profiles (Fig. 1E) showed 15 spicy-related compounds identified (Table S5) that were potentially correlated with the pungent smell of HC, and 14 of them, including ethyl 2-furan propionate, myrtenal, and beta-guaiene etc., were increased after the bleaching. Two sulfur-containing metabolites furfuryl thioacetate and 2-methyl-1,3-dithiacyclopentane, were found to increase in bleached HC samples significantly and could be related to its sulfur odor.

3.3. Screening and identification of chemical bleaching markers of HC

3.3.1. Screening of potential bleaching markers with UPLC/Q-TOF MS

The T-0 and T-1 samples were employed to screen the potential bleaching markers in HC using UPLC/Q-TOF MS-based metabolomic methods. The base peak chromatograms (BPC) showed (Fig. 3A) noticeable changes in the chemical profiles of HC samples before and after bleaching. In multivariate statistical analysis, T-0 and T-1 groups were separated in the PCA score plot (Fig. 3B), indicating a significant change in their chemical compositions. Then, an OPLS-DA ($R^2Y = 0.998$, $Q^2 = 0.995$, Fig. 3C) and volcano plots (Fig. 3D) were generated to characterize the significant differential metabolites further. Finally, 34 variables with VIP > 6 and $p < 0.05$ were selected (Table S6), including 25 with significantly increased signal response after bleaching and 9 with decreased signal response (Fig. 3E). Based on the neutral loss of H_2SO_3 and SO_3 (Wu et al., 2022) from sulfonated components observed in mass spectrometry, seven potential chemical bleaching markers that emerged post-bleaching were identified (Table 1). The identification process of typical examples of peaks 7 and 23 are shown below.

For peak 7 (Fig. 4A), first, the *m/z* 435.06 Da $[M-H]^-$ in MS1 indicated the formula as $C_{16}H_{20}O_{12}S$. Then, the fragments ions *m/z* 353.09, 191.06, 179.03, and 135.04 Da detected in MS2 can be attributed to the $[M-H]^-$ ion lost one molecule of H_2SO_3, $C_9H_8O_4$, $C_7H_{10}O_5$ and CO_2, respectively. Next, the fragment ion *m/z* 353.09 Da indicated a formula as $C_{16}H_{18}O_9$. Its fragmentation behavior was compared with the reference and was presumed to be the chlorogenic acid. Accordingly, the compound of peak 7 was believed to be the product of an additional reaction of chlorogenic acid with H_2SO_3.

For peak 23 (Fig. 4B), the major ions *m/z* 279.13 Da $[M-H]^-$ and *m/z* 599.26 Da [2 M-H]$^-$ in MS1 indicated its formula as $C_{12}H_{24}O_5S$. The fragments ions *m/z* 197.15 Da $[M-H_2SO_3]^-$, 153.13 Da $[M-C_2H_4O-H]^-$, and *m/z* 80.97 Da $[M-C_{12}H_{22}O_2-H]^-$ were detected in MS2. Then, the ion *m/z* 197.15 Da $[M-H_2SO_3]^-$ indicated a formula of $C_{12}H_{22}O_2$ and was identified as houttuynin after comparing the mass information with the reference. Finally, peak 23 was inferred to be the product of an addition reaction of houttuynin with H_2SO_3. Intriguingly, as peak 23 was the most obviously increased component after bleaching treatment (Fig. 3A), it could be potentially used as the marker for the identification of HC bleached with sodium metabisulfite. Accordingly, a preparation, isolation, and absolute structure identification of peak 23 was further performed.

3.3.2. Preparation, isolation, and structure identification of marker peak 23

According to the method described in 2.5, the monomer compound of peak 23 was prepared and isolated, and its structure was identified in an integrated manner. The structural information of the product and its reference substance is indicated in Table 2 and Table S7.

The UV absorption spectra showed that the maximum absorption wavelength of the prepared product was 283.0 nm, which was consistent with the sodium houttuyfonate standard substance (Fig. S4A–B), indicating that the prepared product had the same conjugated system structure with sodium houttuyfonate under the same mobile phase system. It was also observed in MS that the prepared product had the same molecular weight as sodium houttuyfonate, and their fragmentation behavior was also consistent (Fig. S4C–D). Moreover, the prepared product's NMR data (Fig. S5) were resolved and compared with the standard substance. The results showed that the chemical shifts of the carbon and hydrogen atoms at the C1, C2, C3, and C4 positions of the prepared product were shifted compared to the standard substance. However, other positions were the same. It was inferred that as the prepared product was in the free state, the chemical environment near the salt-forming site was changed compared with the reference in the salt-forming state. This led to the change of chemical shifts of carbon and hydrogen atoms near the salt-forming position. While the atoms at other positions were far from the salt-forming position, the chemical environment was less changed. Therefore, the chemical shift values were the same with reference. In summary, the structure of this compound was finally determined as revealed in Table 2, and was named 1-hydroxy-3-oxodecane-1-sulfonic acid ($C_{12}H_{24}O_5S$).

3.3.3. Identification of bleached commercial HC samples using marker peak 23

To verify the efficiency of the obtained marker 1-hydroxy-3-oxododecane-1-sulfonic acid (Peak 23), 15 batches of commercial HC samples were detected and identified with the marker (*m/z* 279.13 Da in negative mode). Results indicated that two samples were found to be bleached (Fig. S6). Furthermore, the sulfur dioxide residues (Table S1) were also detected only in the *No.*2 sample (157.71 mg/kg) and *No.11* sample (203.31 mg/kg). This verification result showed that 1-hydroxy-3-oxododecane-1-sulfonic acid could be an effective marker to evaluate whether HC was bleached. Additionally, although the marker was prepared from sodium metabisulfite bleached HC sample, because of the similar working principle of sulfite food additives (Martínez-Blay et al., 2021), this marker would remain valid if other sulfates like potassium metabisulfite, aluminum sulfate, and aluminum potassium sulfate were used rather than sodium metabisulfite.

A quantitative analysis method of the marker peak 23 was also developed. The method was first validated, and the results showed that

Table 1
The seven potential chemical markers identified in bleached HC samples.

Peak *No.*	Rt (min)	$[M-H]^-$	Formula	Error (ppm)	Major fragment ions (*m/z*)	Prototype compound
Peak 1	1.61	379.0703	$C_{14}H_{20}O_{10}S$	−0.1	107.0499, 135.0447, 161.0462, 297.0984	Ameliaroside
Peak 4	1.99	435.0596	$C_{16}H_{20}O_{12}S$	0.8	135.0448, 179.0347, 191.0554, 261.0072, 353.0871, 435.0598	Neochlorogenic acid
Peak 5	2.26	217.0176	$C_8H_{10}O_5S$	−1.1	65.0394, 77.0397, 106.0415, 135.0457	*β*-Myrcene
Peak 7	2.30	435.0601	$C_{16}H_{20}O_{12}S$	−0.4	135.0448, 161.0243, 179.0346, 191.0554, 261.0075, 353.0873	Chlorogenic acid
Peak 10	5.27	283.0391	$C_{11}H_{12}N_2O_5S$	−1.1	79.9573, 116.0510, 142.0661, 222.0225,	Tryptophan
Peak 18	15.28	383.0843	$C_{13}H_{20}O_{13}$	1.8	83.0854, 138.0319, 237.1495, 301.1126, 319.1205	Quercetin
Peak 23	17.26	279.1276	$C_{12}H_{24}O_5S$	−3.1	81.1232, 81.4502, 197.1504	Houttuynin

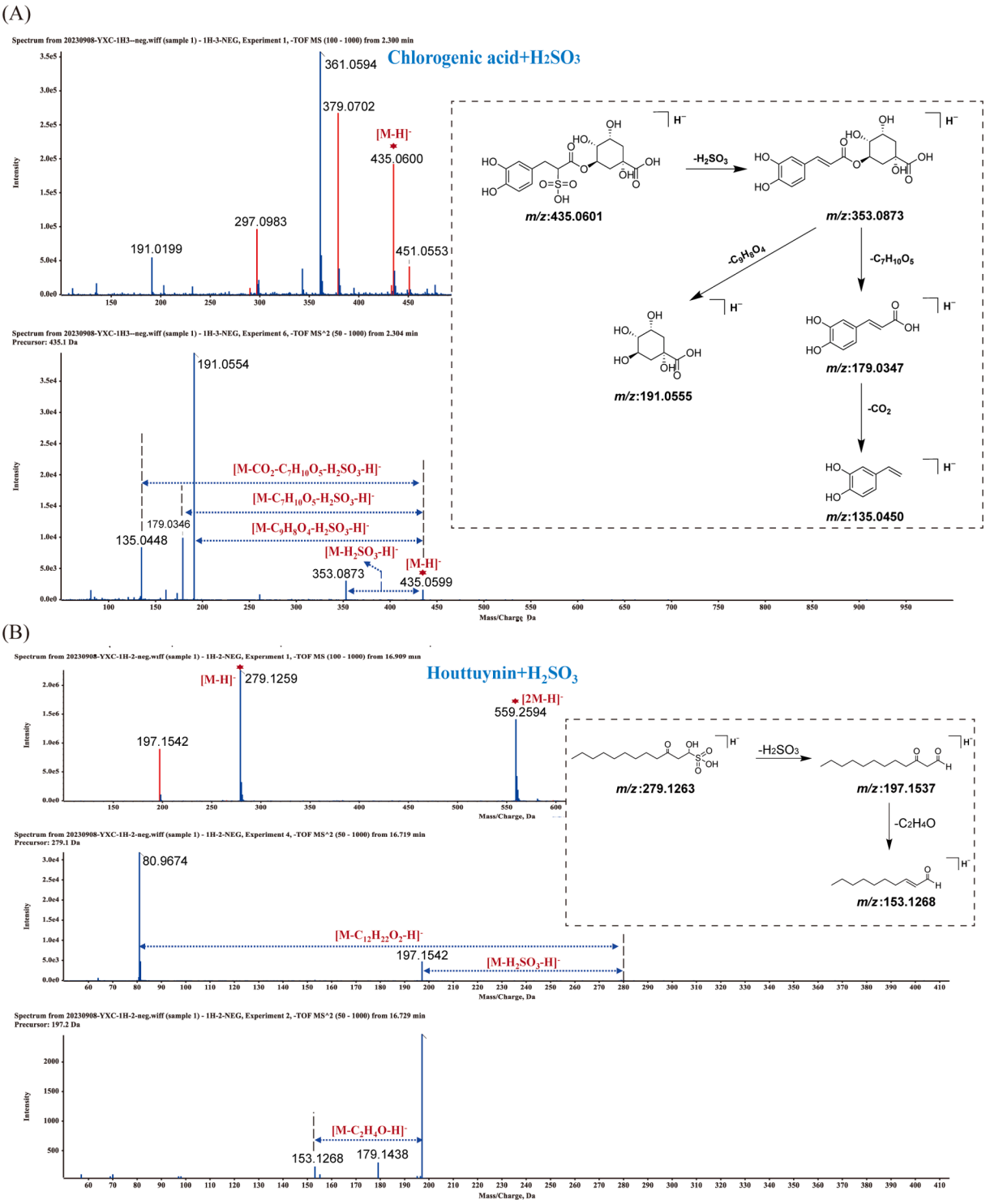


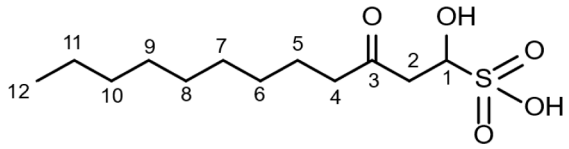
Fig. 4. Examples of the structure identification of potential chemical bleaching markers in HC with MS/MS data. (A) Marker peak 7. (B) Marker peak 23.

the calibration curve $Y = 5.9975X + 1.7189$ (r = 0.9995) displayed an optimal linear relationship within 1.52 to 97.38 μg/mL. The LOD and LOQ were 0.19 and 0.38 μg/mL, respectively. The RSD of the peak areas was 0.11 % in the precision test, 1.35 % in the repeatability test, 0.95 % in the stability test, and the recovery was 99.92 % with an RSD of 2.67 % (Table S8). All these results demonstrated that the method was applicable to the quantification of the marker compound. The determination results of HC samples showed that the marker content in bleached HC samples increased with the treatment time (Fig. S7). Its changing trend showed a rapid increase at the beginning of bleaching, and then the growth rate slowed down and tended to stabilize. After 1 h of bleaching, the content of 1-hydroxy-3-oxododecane-1-sulfonic acid in T-1 samples reached about 28 mg/g, and for T-8, it was approximately 42 mg/g.

4. Conclusion

In this study, the HC samples bleached with sodium metabisulfite demonstrated noticeable bleaching and preservation effects. The study analyzed the overall non-volatile and volatile metabolites of HC samples using LC-MS and GC-MS techniques. Significant changes in the chemical

Table 2
The structure information of the prepared marker peak 23.

	Peak 23
Structure	
PDA absorption maximum	283.0 nm
MS fragment in ESI-	*m/z* 279, 197, 80.9
1H NMR (400 MHz, DMSO-d_6)	0.85(3H, t, J = 6.6 Hz, H-12), 1.46(2H, m, H-5), 1.24(12H, m, H6-11), 2.48(1H, H-2a), 2.36(1H, H-2b), 5.47(1H, m, H-1)
^{13}C NMR (100 MHz, DMSO-d_6)	13.91(C-12), 22.06(C-11), 23.27(C-10), 28.62(C-9), 28.66(C-8), 28.86(C-7), 28.89(C-6), 31.24(C-5), 43.03(C-4), 198.63(C-3), 55.81(C-2), 80.88(C-1)

composition of HC before and after bleaching were characterized through widely targeted metabolomics analysis, revealing a positive correlation between the extent of changes in chemical composition and the duration of bleaching. The most notable changes mainly occurred at the initial stages of bleaching. Subsequently, an untargeted UPLC/Q-TOF MS method was employed to explore the potential chemical bleaching markers in bleached HC samples, and seven sulfur compounds were identified. Lastly, the marker peak 23, which exhibited the most significant change and highest abundance among the potential markers, was further prepared and isolated. Its structure was identified as 1-hydroxy-3-oxodecane-1-sulfonic acid with NMR. The bleaching marker of HC was verified in market HC samples with sulfur dioxide residue analysis as a reference. The results showed that this marker could be used to identify bleached HC effectively. As HC is widely used as a food and both herbal and pharmaceutical ingredient, although sulfites are common food additives used in agricultural products (Ren et al., 2022), the effect of bleaching on the quality of HC found in this study indicated that people should remain vigilant about the use of HC bleached with sulfites. This work is a valuable case for the recognition of the processing of agricultural products with sulfites from the perspective of quality impact, especially when used for medicinal purposes.

CRediT authorship contribution statement

Dan Zhao: Methodology, Investigation. **ChangGui Yang:** Resources, Investigation. **ChengHong Xiao:** Investigation. **Tao Zhou:** Formal analysis. **DeHua Wu:** Software. **Sheng Wang:** Resources. **ChuanZhi Kang:** Data curation. **LanPing Guo:** Funding acquisition. **Ye Yang:** Writing – review & editing, Supervision. **ChaoGeng Lyu:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101463.

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