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Comprehensive metabolomic variations of hawthorn before and after insect infestation based on the combination analysis of ^{1}H NMR and UPLC-MS

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ABSTRACT

Hawthorn, the sliced and dried ripe fruits of *Crataegus pinnatifida* Bge. Var. *Major* N. E. Br. (Rosaceae), is an edible and medicinal substance with a variety of health-promoting benefits. Hawthorn needs to be stored in warehouses after harvesting to meet people's perennial demand. However, it is easily infested by insects of *Plodia interpunctella* and *Tribolium castaneum* during storage, which inevitably leads to poor quality and causes adverse effects on people's health. So far, there has been no report on insect-infested hawthorn. In this study, we analyzed the changes of metabolites in hawthorn before and after insect infestation and screened out potential biomarkers to effectively and quickly detect the occurrence of insect infestation. A combination analysis of ^{1}H nuclear magnetic resonance (NMR) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) was used to identify the primary and secondary metabolites. By the comparison of hawthorn and insect-infested hawthorn samples, it was found that the differences were mainly manifested in the content of metabolites. The metabolites of 32 and 1463 were identified by ^{1}H NMR and UPLC-MS analysis, respectively. According to the parameters of VIP >1 and $P < 0.05$, 10 differential metabolites were screened from ^{1}H NMR analysis. Based on the parameters of VIP >1.0, $P < 0.05$, and (FC) > 1 or < 1, 47 differential metabolites were screened from UPLC-MS analysis. Therefore, a total of 57 differential metabolites were considered as differential biomarkers. The heat map analysis showed that the content of some differential biomarkers with significant pharmacological activities decreased after insect infestation. Through receiver operating characteristic (ROC) curve assessment, 52 differential biomarkers (6 of ^{1}H NMR analysis and 46 of UPLC-MS analysis) were screened to distinguish whether insect infestation occurred in hawthorn. This is the first report on the changes of metabolites between hawthorn and insect-infested hawthorn and on the screening of differential biomarkers for monitoring insects. These results contributed to evaluate quality of hawthorn and ensure food safety for consumers. It also laid a foundation for further research on the infestation mechanism and safe storage monitoring in hawthorn.

1. Introduction

The plant of *Crataegus pinnatifida* Bge. Var. *Major* N. E. Br. (family Rosaceae) is a fruit tree widely distributed in Asia, Europe, North Africa, and North America (Wang et al., 2023a; Wen et al., 2015). Its fresh ripe fruits are picked on, purified, sliced, and dried in the sunlight to obtain edible and medical hawthorn. It has been reported that more than 150 bioactive substances, including flavonoids, phenolic acids, organic acids, lignans, terpenoids, and sugars, have been found in hawthorn, especially rich in flavonoids and phenolic acids (Wang et al., 2023a). As one of herbal raw material, hawthorn has been officially documented in Chinese, European, American, and Canadian Pharmacopeias (Wang et al., 2023a; Zureke et al., 2021). A lot of reports indicate that hawthorn has effects of anti-inflammatory, anti-ulcer, anti-virus, anti-anxiety, anti-oxidant, reducing lipid, and regulating blood sugar. Besides, hawthorn is also used to treat kidney stones, cardiac diseases, liver diseases, and central nervous system diseases (Kumar et al., 2019; Wang et al., 2023a).

Due to the pleasant flavor, fascinating color, and rich nutrition, hawthorn has attracted great interest among consumers as a healthy food. It can be eaten directly and processed into jellies, jams, candies, juice, and wine (Zhang et al., 2020). The ripening and harvesting of hawthorn is seasonal, but its consumption is continuous. In order to meet the demand of consumers, a large number of hawthorns need to be stored in warehouses after being harvested. The *Plodia interpunctella*

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Abbreviation

PI-hawthorn *P. interpunctella* infested hawthorn
TC-hawthorn *T. castaneums* infested hawthorn

(Hübner) (Lepidoptera: Pyralidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) are two primary injurious insects for stored food products, including various grains, dried fruits, pulses, and dried vegetables. After being infested, these food products are contaminated with excreta, dead bodies, and other secretions of insects, resulting in a serious reduction in nutritional value, quality, and safety level (Gao et al., 2022; Zhao et al., 2022; Zhou et al., 2022). According to our previous investigation on the quality of hawthorn, it was easily infested by *P. interpunctella* (PI) and *T. castaneum* (TC) during storage, due to inappropriate storage conditions and management measures. The insect infestation not only caused losses in terms of quantity of hawthorn, but also affected on quality by depleting nutrients and contamination with excreta and secretions. Severe insect infestation could produce carcinogenic components, which had adverse effects on people's health (Kumar et al., 2020). Therefore, insect-infested hawthorn was unhygienic, making it unfit for consumption directly or processing into food products. However, a few unscrupulous companies often used the insect-infested hawthorn for deep processing of food products. Traditional methods such as visual inspection, probe sampling, trapping, and microscopy analysis are commonly used to dynamically determine the occurrence of insect infestation, but these disadvantages of time-consuming, labor-intensive, low efficiency, and human subjectivity are prominent (Tian et al., 2022; Srivastava et al., 2018). Furthermore, they cannot be used to detect processed food products. Therefore, it is important to study the changes of metabolites in hawthorn before and after insect infestation and to screen biomarkers for monitoring insects. At present, some reports on hawthorn mainly focus on the differences of physical properties and metabolites from different sources and at different maturation stages, as well as the activities of regulating intestinal lipid metabolism, bacteriostasis, and antioxidation (Li et al., 2015; Kumar et al., 2018; Lund et al., 2017; Wen et al., 2015). So far, there have been no studies on insect-infected hawthorn.

As a systematic, powerful, and efficient method, metabolomics analysis can investigate changes in metabolites by identifying the fingerprints (Feng et al., 2022; Wang et al., 2023b). In general, plant metabolites can be divided into primary metabolites and secondary metabolites. The former provides nutrients for plant growth and development and the latter is used against pathogens or competitors (Kumar et al., 2019). The nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS) are two important analytical methods for the detection of metabolites and have been widely applied on the biology, food, and medicine fields (Li et al., 2022). NMR method has the advantage on identifying abundant primary metabolites of plant in a single spectrum (Dai et al., 2010) and has been used to analyze the changes of metabolites for cold-stored strawberry, postharvest sweet cherry fruit, and the polar and non-polar extracts of persimmon (Maulidiani et al., 2018; Huang et al., 2022; Goulas et al., 2015). LC-MS is a fast and sensitive analytical method to focus on the secondary metabolites (Zhang et al., 2018) and has been used to analyze the metabolic variation of Chenpi and Qingpi with different storage years, and oriental strawberry before and during postharvest storage (Yang et al., 2022; Olennikov et al., 2022; Cheng et al., 2023a). In order to obtain comprehensive and complementary information of primary and secondary metabolites, the combination analysis of ^{1}H NMR and LC-MS was used to analyze hawthorn before and after insect infestation in this study.

Normal hawthorn samples were considered as a control group. In order to exclude the influence of insect factors, the infested hawthorn samples by TC and PI were set as two experimental groups. The combination analysis of ^{1}H NMR and UPLC-MS methods coupled with chemometrics were used to comprehensively investigate the changes of metabolites and to screen differential biomarkers with the purpose of clarifying the differences of hawthorn before and after insect infestation. These results aimed to quickly identify the insect-infested hawthorn and served as a platform for further study on infestation mechanisms and safe storage monitoring.

2. Materials and methods

2.1. Chemical and regents

Analytical grade methanol, ethanol, petroleum ether, and ethyl acetate were purchased from Tianjin Fuyu Fine Chemical Co., Ltd. (Tianjin, China). Methanol-d_4 (CD_3OD, 99.8%) with 0.03% trimethylsilyl (TMS), dimethyl sulfoxide-d_6 (DMSO-d_6), and deuterium oxide (D_2O) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). MS-grade acetonitrile, formic acid, methanol, and isopropanol were supplied by Thermo Fisher (Osterode, Germany).

2.2. Sample collection

The fresh ripe fruits of *C. pinnatifida* Bge. Var. *Major* N. E. Br. (family Rosaceae) were picked on 25, September 2022 from Fei County, Shandong Province, China (35°39′ N, 118°01′ E, altitude 120 m). Then, these operations of purifying, slicing, and sun drying were carried out in our laboratory to obtain a basic material of hawthorn, an edible and medicinal substance. In these processes, some safe measures such as insect control, mildew prevention, and rain prevention were adopted to avoid environmental contamination. The plant was identified by Prof. Zhimao Chao (Institute of Chinese Materia Medica, China Academy of Chinese Medical Science) according to the description in Flora of China database (http://www.iplant.cn/foc/, accessed on 26 September 2022). The voucher specimens (No. CF20220925) were deposited at 1022 laboratory of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Science, Beijing, China.

2.3. Infestation process of insects

A total of 100 larval insects of PI were transferring to a transparent cubic jar with 9 cm length of the side. Hawthorn samples of 40 g were added into the cubic jar and placed for 30 d to carry out the PI infested hawthorn (PI-hawthorn) samples. A total of 250 adult insects of TC was transferring to a same size jar with 40 g hawthorn samples for 30 d to carry out TC infested hawthorn (TC-hawthorn) samples. Besides, the hawthorn samples of 40 g were placed in same size jar as a control group. Every jar was equipped with 150 mesh gauze and fixed using rubber band for preventing insects from getting out and running into. The infestation process was performed on an HQ-250 incubator (Zensan Electrical Technology Co., Ltd., Shanghai, China) with temperature at 30 °C and relative humidity (RH) of 65%. Each infesting experiment was repeated four times. All insects of PI and TC were fed at the conditions of 30 °C and 65% RH in total darkness before being used. The insect-infested hawthorn samples were obtained by removing the insect excreta and dead bodies using brushes and sieves. The appearance traits of hawthorn and insect-infested hawthorn (PI-hawthorn and TC-hawthorn) samples were shown in Fig. 1. All of these materials were crushed into powder samples using a DFT-50 A pulverizer (Linda Machinery Co. Ltd., Hangzhou, China) and sieved through 65 mesh.

2.4. Samples preparation

2.4.1. ^{1}H NMR samples

The preparation of ^{1}H NMR samples was described references with minor modifications (Xu et al., 2021). The powder samples were

Fig. 1. The appearance traits of hawthorn, PI-hawthorn, and TC-hawthorn samples. PI-hawthorn: *P. interpunctella* infested hawthorn; TC-hawthorn: *T. castaneums* infested hawthorn.

weighed accurately (1.0 g), put into a 50 mL centrifuge tube, mixed with 25 mL extraction solvent (Table 1), and weighed. The mixture was vortexed for 1 min, extracted ultrasonically for 30 min (100 W, 40 kHz) using a KQ-100 E ultrasonicator (Kunshan Ultrasonic Instruments Co. Ltd., Kunshan, China), cooled to room temperature at 25 °C, weighed again, and made up for lost weight with corresponding extraction solvent. The extract was centrifuged at 3500 rpm for 10 min at 4 °C. The supernatant was transferred into 35 mL round-bottomed flasks, concentrated with a rotary vacuum evaporator under reduced pressure at 50 °C, and mixed well with 1.0 mL D_2O. The mixed solution was steamed on a water bath at 60 °C, dried with P_2O_5 for 3 d, resolved with 1.0 mL deuterated solvent (Table 1), and filtrated with 0.22 μm membrane filter. The filtrate of 0.6 mL was transferred into nuclear magnetic tube (Wilmad-LabGlass, NJ, USA) for ^{1}H NMR analysis.

2.4.2. UPLC-MS samples

The powder samples were weighed accurately (50 mg), put into 2 mL centrifuge tube, added into 400 μL methanol-water (4:1, *v/v*) solution, ground for 6 min at a freezing temperature at −10 °C, extracted ultrasonically for 30 min (100 W, 40 kHz), and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was filtrated through a 0.22 μm membrane filter and transferred to a sample vial for UPLC-MS analysis. The quality control (QC) samples were obtained by pooling a 20 μL aliquot of all analyzed sample solutions.

2.5. Metabolite analysis conditions

2.5.1. ^{1}H NMR conditions

The ^{1}H NMR analysis were performed on a Bruker AVANCE II 400 MHz spectrometer (Bruker, Billerica, MA, USA) at 293 K. A zg30 pulse sequence was employed to suppress the residual water signal. TMS and CD_3OD provided chemical shift calibration at δ 0.00 ppm and field frequency locking at δ 3.31 ppm, respectively. For each sample, 16 transients were collected using a spectral width of 8012 Hz, an acquisition time of 4.09 s, and a relaxation delay of 1.00 s. All free induction decays (FIDs) were multiplied by an exponential function with a 0.3 Hz line broadening factor prior to Fourier transformation.

2.5.2. UPLC-MS conditions

The UPLC-MS analysis were carried out on a UPLC-Q Exactive HF-X system (Thermo Fisher Scientific, MA, USA). Metabolites were separated using a Waters ACQUITY UPLC HSS T3 chromatographic column (100 mm × 2.1 mm i. d., 1.8 μm, Waters, Milford, USA) with column temperature at 40 °C and injection volume of 3 μL. The mobile phase A was composed of 95% water (containing 0.1% formic acid) and 5% acetonitrile and mobile phase B was 47.5% acetonitrile, 47.5% isopropanol, and 5% water (containing 0.1% formic acid). The flow rate of mobile phase was set at 0.4 mL/min. The gradient elution program of positive ion mode was as follows: 0–3.0 min for 0–20% B, 3.0–4.5 min for 20%–35% B, 4.5–5.0 min for 35–100% B, 5.0–6.3 min for 100% B, 6.3–6.4 min for 100%–0% B, and 6.4–8.0 min for 0% B. The gradient elution program of negative ion mode was 0–1.5 min for 0–5% B, 1.5–2.0 min for 5%–10% B, 2.0–4.5 min for 10%–30% B, 4.5–5.0 min for 30%–100% B, 5.0–6.3 min for 100% B, and 6.3–8.0 min for 0% B.

For the MS condition, the positive and negative ion scanning modes of electrospray ionization (ESI) source were used to collect the mass spectrum signals. The spray voltage was set at (+) 3500 and (−) 3500 V. The mass range was *m/z* 70 to 1050. The flow rates were set at 50 arb for sheath gas and 13 arb for aux gas. Full MS resolution was 60,000 and MS/MS resolution was 7500. The Data Dependent Acquisition mode was used to collect data. The QC sample was performed every four samples to ensure the stability of analytical system and the reliability of data. The injection volume and detection method of QC samples were consistent with those of the experimental samples.

Table 1
Different solvent systems of sample preparation in ^{1}H NMR analysis.

No.	Extraction solvent	Solvent polarity	Deuterated solvent
E1	petroleum ether	Weak	DMSO-d_6
E2	ethyl acetate	Medium	CD_3OD
E3	ethyl acetate	Medium	DMSO-d_6
E4	Methanol	Strong	CD_3OD
E5	70% ethanol	Strong	CD_3OD
E6	70% ethanol	Strong	DMSO-d_6

2.6. Pre-processing for data analysis

2.6.1. ^{1}H NMR data analysis

All FIDs files were imported into MestRenova 14.0 software (Mestrelab Research, Santiago, Spain), carried out automatic phase and baseline correction, and calibrated to δ 0.00 ppm (the proton signal of TMS) of spectra. The ^{1}H NMR data were segmented into regions of 0.04 ppm width corresponding to the total region of δ 9.0–0.0 ppm. Meanwhile, the regions of δ 3.38–3.28 ppm corresponding to residual CD_3OD signal and δ 5.08–4.75 ppm corresponding to imperfect D_2O suppression were discarded. All remaining spectral segments were normalized to the sum of spectra for reducing variations resulted from the concentration inconsistency before chemometrics analysis (Li et al., 2018; Xu et al., 2021; Zhao et al., 2019). The metabolites were identified by consulting literatures and HMDB (http://www.hmdb.ca/) according to chemical shifts, peak areas, and multiplicity.

2.6.2. UPLC-MS data analysis

The collected raw data were imported into Progenesis QI v2.3 software (Nonlinear Dynamics, Newcastle, UK) for baseline filtering, peak identification, integration, retention time correction, peak alignment, peak extraction, and normalization (Cheng et al., 2023a). Next, some data including retention time, *m/z*, and chromatographic peak intensity were derived. The identification of metabolites was based on biochemical databases, such as the Human Metabolome Database (https://www.

hmdb.ca/) (Li et al., 2022).

2.7. Multivariate data analysis

The two data sets resulting from ^{1}H NMR and UPLC-MS were further analyzed by chemometrics including principal component analysis (PCA), partial least squares discriminate analysis (PLS-DA), and orthogonal partial least squares discriminate analysis (OPLS-DA) using SIMCA-P14.0 software package (Umetrics, Umeå, Sweden) and the R ropls package (https://www.bioconductor.org/packages/devel/bioc/vignettes/ropls/inst/doc/ropls-vignette.html). The volcano plot was employed to assist in screening the differential metabolites during UPLC-MS analysis. The heat map with hierarchical clustering analysis (HCA) was used to illustrate the content changes of differential metabolites in different comparison groups. Independent sample *t*-test was used to assess whether the differences were statistically significant ($P < 0.05$). At last, a receiver operating characteristic (ROC) curve analysis was carried out by MetaboAnalyst (https://www.metaboanalyst.ca/) to determine the effectiveness of screened differential metabolites.

3. Results and discussion

3.1. Optimization the preparation conditions of ^{1}H NMR samples

The chemical compounds of different species of hawthorn and their processed products had been analyzed by ^{1}H NMR metabolomics method (Nie et al., 2019; Guo et al., 2019; Lund et al., 2017, 2020; Roman et al., 2021). However, there was no study on the changes of hawthorn before and after insect infestation. In the study, the ^{1}H NMR metabolomics method was firstly used to analyze the differences of metabolites between hawthorn and insect-infested hawthorn (PI-hawthorn and TC-hawthorn) samples.

The metabolomics analysis was recommended to detect metabolites as many as possible in the overall research design (Fan et al., 2012). Therefore, the solvent system with excellent comprehensive extraction ability of metabolites should be chosen during the process of sample preparation. Up to now, it had been reported that several extraction solvents (petroleum ether, ethyl acetate, methanol, ethanol, and water) and deuterated solvents (DMSO-d_6 and CD_3OD) were employed to survey the metabolite differences in plants (Hernández-Guerrero et al., 2021; Igoumenidis et al., 2018; Zhang et al., 2020). Each solvent had its own advantage and application scope. In general, the selection of solvent system was considered to meet the "like-dissolves-like" criterion based on the polarity of targeted metabolites. Therefore, six solvent systems (Table 1) were optimized and their ^{1}H NMR spectra were shown in Fig. S1. Due to the less peak signal information of E1 spectrum and the poorer peak shape of E3 spectrum, their E1 and E3 solvent systems were not considered. Comparing the E2, E4, and E5 solvent systems with same deuterated solvent of CD_3OD, the peak signals of E2 spectrum were more abundant than those of E4 and E5 spectra, so ethyl acetate (E2) was selected as extraction solvent. The E5 and E6 solvent systems had same extraction solvent of 70% ethanol and different deuterated reagents of CD_3OD and DMSO-d_6, respectively. The ^{1}H NMR spectrum of E5 showed more peak signals than that of E6. Therefore, CD_3OD was used as deuterated solvent. Based on the above results, the ^{1}H NMR samples were prepared by the solvent system of ethyl acetate extraction and CD_3OD dissolution. This solvent system could simultaneously extract more metabolite information with a single measurement as desired for meeting the requirements of metabolomics analysis.

3.2. Chemical profiling of hawthorn, PI-hawthorn, and TC-hawthorn

The metabolites of hawthorn, PI-hawthorn, and TC-hawthorn samples were analyzed based on ^{1}H NMR metabolic profiles. A total of 32 metabolites were identified and assigned to amino acids, sugars, phenolic acids, and flavonoids. All identified metabolites were carefully compared with the standards, databases and earlier reports (Nie et al., 2019; Xu et al., 2021; Yan et al., 2020). Their detailed information were shown in Table S1. The representative ^{1}H NMR spectra of hawthorn, PI-hawthorn, and TC-hawthorn samples were shown in Fig. 2. Each ^{1}H NMR spectrum was approximately divided into three different regions, consisting of phenolic acids and flavonoids region (δ 9.00–6.00 ppm), sugars region (δ 6.00–3.50 ppm), and amino acid region (δ 3.50–0.00 ppm). It could be found that hawthorn, PI-hawthorn, and TC-hawthorn samples had similar profile on a whole, but several differences on signal intensity were highlighted in red boxes (Fig. 2) by visual inspection. In the region of δ 7.40–5.80 ppm, the overall intensity of peak signals in hawthorn samples was higher than that in insect-infested hawthorn samples. For example, the intensity of procyanidin B2 (δ 6.76, d; δ 6.80, d) in hawthorn samples was higher than that in other two samples. These results shown that phenolic acids and flavonoids were more in hawthorn samples. The strong peak signals locating in the region of δ 4.70–3.50 ppm were attributed to sugars. As shown in Fig. 2, the peak signals of β-D-glucose (δ 4.50, d) and β-galactose (δ 4.49, d) in hawthorn samples were higher than those in PI-hawthorn and TC-hawthorn samples. The result shown that the content of two sugars is higher in hawthorn samples than that in insect-infested hawthorn. In the range of δ 3.50–0.00 ppm, the peak signals of aspartic acid (δ 2.66, dd; δ 2.81, dd) in hawthorn samples were higher than those in PI-hawthorn and TC-hawthorn samples. The result shown that the content of aspartic acid was higher in hawthorn samples than that in insect-infested hawthorn. Based on these ^{1}H NMR results, some primary metabolites and part of secondary metabolites were identified from hawthorn, PI-hawthorn, and TC-hawthorn samples. The peak signal intensity of metabolites indicated that their content had differences before and after insect infestation.

LC-MS metabolomics method is of great significance in phytochemical analysis, because the key secondary metabolites can be better separated and detected (Yan et al., 2020). After peak extraction, deconvolution, alignment, and normalization, UPLC-MS spectra of hawthorn, PI-hawthorn, and TC-hawthorn samples were acquired by switching positive and negative scan modes (Fig. S2). A total of 1463 metabolites (Table S2) were identified and tentatively characterized. The identified metabolites included flavonoids, phenylpropanoids, amino acids related compounds, fatty acids related compounds, alkaloids, terpenoids, shikimate/acetate-malonate pathway derived compounds, polyketides, and others. Their detailed classification was exhibited in Fig. S3.

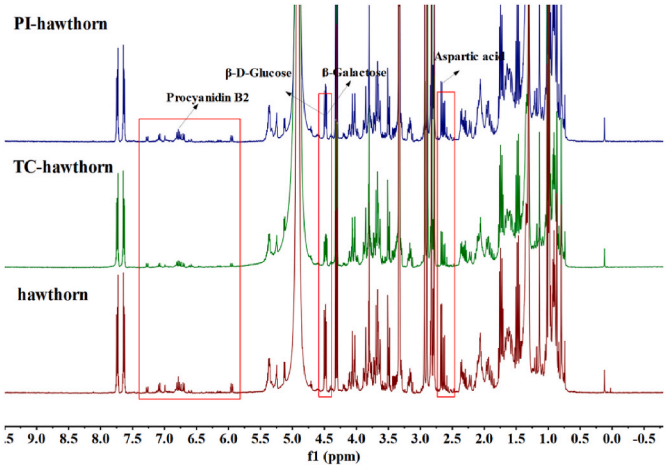

Fig. 2. The representative ^{1}H NMR spectra of hawthorn, PI-hawthorn, and TC-hawthorn samples. Several metabolite differences on signal intensity were highlighted in red boxes by visual inspection. PI-hawthorn: *P. interpunctella* infested hawthorn; TC-hawthorn: *T. castaneums* infested hawthorn. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In short, the abundant primary and secondary metabolites of hawthorn before and after insect infestation were identified based on the combination analysis of ^{1}H NMR and UPLC-MS metabolomics methods. These spectra showed that insect infestation could cause metabolite changes, and these changes were mainly showed in the content of metabolites rather than in the level of appear or disappear. The ^{1}H NMR focused on the detection of the primary metabolites. UPLC-MS results mainly showed abundant secondary metabolites. Therefore, the combination analysis of ^{1}H NMR and UPLC-MS methods could obtain complementary metabolite information and was a very powerful strategy to

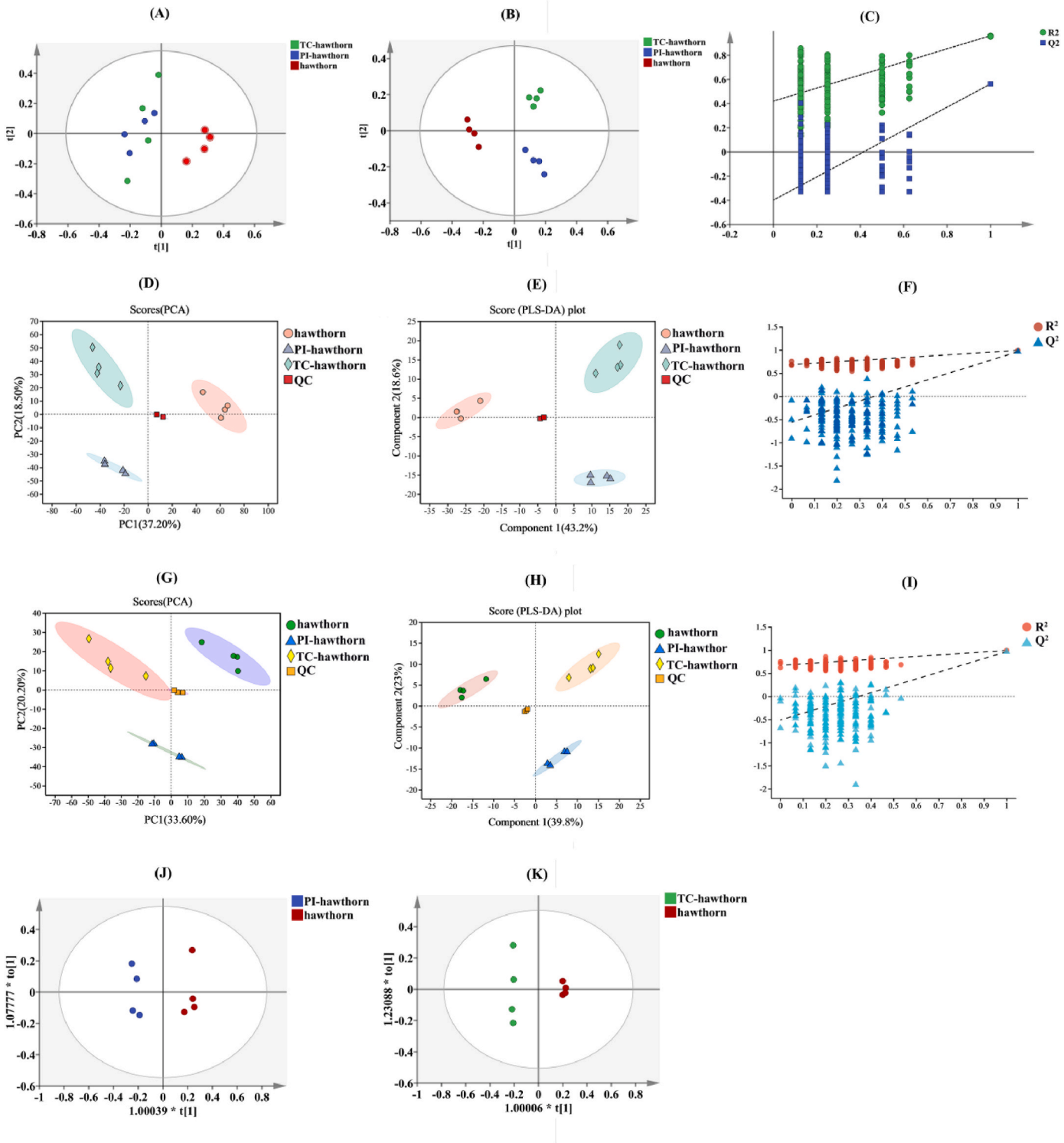

Fig. 3. Multivariate statistical analysis of hawthorn metabolite information. The score plots of PCA (A), PLS-DA (B), and permutation test of 200 times (C) of hawthorn, PI-hawthorn, and TC-hawthorn samples based on ^{1}H NMR analysis. The score plots of PCA (D), PLS-DA (E), and permutation test of 200 times (F) in positive ion mode and PCA (G), PLS-DA (H) and permutation test of 200 times (I) in negative ion mode of hawthorn, PI-hawthorn, and TC-hawthorn samples based on UPLC-MS analysis. The OPLS-DA score plots of hawthorn *vs* PI-hawthorn group (J) and hawthorn *vs* TC-hawthorn group (K) based on ^{1}H NMR analysis. PI-hawthorn: *P. interpunctella* infested hawthorn; TC-hawthorn: *T. castaneums* infested hawthorn.

analyze the metabolite changes of hawthorn and insect-infested hawthorn.

3.3. Unsupervised and supervised data analysis

Both ^{1}H NMR and UPLC-MS spectra provided a large amount of fingerprint information. But the reflected information was quite complex and difficult to determine the differential metabolites before and after insect infestation by visual inspection alone. In order to clearly compare differences and screen differential metabolites between hawthorn and insect-infested hawthorn, chemometrics analysis was applied including PCA, PLS-DA, and OPLS-DA. PCA, an unsupervised analysis, is widely used for feature extraction, dimensionality reduction, and samples classification. Both PLS-DA and OPLS-DA, the supervised analysis, have better discriminant ability for the samples with large within-class differences than PCA (Zhang et al., 2023).

The PCA score plot of ^{1}H NMR (Fig. 3A) displayed a classification trend between hawthorn and insect-infested hawthorn (PI-hawthorn and TC-hawthorn). The first two principal components (PCs) were 31.5% and 25.3%, respectively. All points were scattered in the ellipse (95% of Hotelling's T2), indicating no abnormal samples existing. As shown in Fig. 3A, there was no overlap between hawthorn and insect-infested hawthorn samples. This result indicated that metabolites had significant differences in hawthorn before and after insect infestation. It was worth noting that both PI-hawthorn and TC-hawthorn samples existed overlap, indicating that certain metabolites of hawthorn kept the same change trend after insect infestation. These metabolites with the same trend could be labeled as biomarkers to distinguish hawthorn and insect-infested hawthorn. The PLS-DA model was further constructed (Fig. 3B) and validated using a permutation test of 200 times (Fig. 3C). All model parameters were more than 0.5 with 0.659 of R^2X, 0.961 of R^2Y, and 0.721 of Q^2. These results indicated that the established PLS-DA model was reliable (Zhang et al., 2023). In the PLS-DA score plot (Fig. 3B), hawthorn, PI-hawthorn, and TC-hawthorn samples had a good separation, which showed significant intergroup metabolite differences. Meanwhile, it revealed a great predictability and good fitting of PLS-DA model, because both R^2 and Q^2 values were lower than original ones (right) of Fig. 3C (Zhang et al., 2023).

In the process of LC-MS metabolomics analysis, the verification of data quality is an important step and the high-quality and reliable data from QC samples were required (Cheng et al., 2023a). As shown in Fig. 3D and G, all QC samples were clustered together and distributed near the origin of coordinates. Based on this, the system was stable and the subsequent outcome analyses were credible. Three clusters revealed that hawthorn, PI-hawthorn, and TC-hawthorn samples could be clearly separated under positive and negative ion modes (Fig. 3D and G). The first three PCs explained the majorities of the variation with PC1 of 37.2%, PC2 of 18.5%, and PC3 of 8.4% for positive ion mode, and PC1 of 33.6%, PC2 of 20.2%, and PC3 of 9.5% for negative ion mode. For evaluating the validity of UPLC-MS metabolomics analysis, the PLS-DA model (Fig. 3E and H) also was established in positive and negative ion modes along with the permutation test of 200 times (Fig. 3F and I). The established PLS-DA model showed good fitting (R^2X 0.759 and R^2Y 0.992) and predictability (Q^2 0.966) for positive ion mode, as well as R^2X 0.760, R^2Y 0.994, and Q^2 0.967 for negative ion mode.

Based on the above results, the combination analysis of ^{1}H NMR and UPLC-MS was valid and satisfactory, which provided a reliable base for screening differential metabolites in hawthorn and insect-infested hawthorn samples.

3.4. Analysis of differential biomarkers

The OPLS-DA model is applied to compare and classify the samples in pairs and the obtained variable importance in projection (VIP) value represents the contribution of each metabolite to the classification. The larger the VIP value, the more significant the classification contribution of metabolite. In general, the value of VIP >1 can be used to screen differential metabolites (Cheng et al., 2023a).

In the process of ^{1}H NMR analysis, the OPLS-DA models of hawthorn *vs* PI-hawthorn group (R^2X 0.744, R^2Y 0.984, and Q^2 0.943) and hawthorn *vs* TC-hawthorn group (R^2X 0.881, R^2Y 0.998, and Q^2 0.957) were constructed to obtain VIP values. As shown in Fig. 3 J and K, the intergroup separation could be apparently observed on hawthorn *vs* PI-hawthorn group and hawthorn *vs* TC-hawthorn group, respectively. The parameters of VIP >1 and $P < 0.05$ were used to obtain differential metabolites. As a result, 17 differential metabolites of hawthorn *vs* PI-hawthorn group and 15 differential metabolites of hawthorn *vs* TC-hawthorn group were screened out. Among them, 10 differential metabolites, including 4 carboxylic acids and derivatives, 3 organooxygen compounds, 2 flavonoids, and 1 organic acid and derivative, were shared. These shared differential metabolites could be considered as differential biomarkers to distinguish hawthorn and insect-infested hawthorn samples.

Potential differential metabolites also can be selected from a volcano plot. In a statistical test, the volcano plot is a scatter map, which can quickly and intuitively screen metabolites with large change amplitudes and statistical significance. The horizontal axis represents the fold change (FC), that is, a ratio of the mean abundance of a metabolite in one group of samples to that in another group of samples. The longitudinal axis represents metabolites with $P < 0.05$. In a volcano plot, the gray dots indicate metabolites without differences. The red and blue dots represent the up-regulated metabolites (FC > 1) and down-regulated metabolites (FC < 1), respectively (Li et al., 2022). Fig. 4A showed a volcanic plot of hawthorn *vs* TC-hawthorn group in positive ion mode. Based on the parameters of $P < 0.05$, VIP >1.0, and (FC) > 1 or < 1, a total of 236 differential metabolites were screened out, of which 135 were up-regulated metabolites (red dots) and 101 were down-regulated metabolites (blue dots). As shown in Figs. S4C and 203 differential metabolites were screened from hawthorn *vs* TC-hawthorn group in negative ion mode using the same screening parameters. Similarly, a total of 456 differential metabolites were screened from hawthorn *vs* PI-hawthorn group, including 254 of positive ion mode and 202 of negative ion mode (Figs. S4A and B). A Venn diagram analysis (Fig. 4B) of differential metabolites showed that 262 differential metabolites were shared in comparison groups of hawthorn *vs* TC-hawthorn and hawthorn *vs* PI-hawthorn. Their classification information was displayed in Fig. 4C. Among them, flavonoids (12.90%), organooxygen compounds (12.50%), and carboxylic acids and derivatives (11.29%) were very abundant metabolites. Then, 47 of 262 shared differential metabolites were characterized (Table S3) and considered as differential biomarkers, including 27 flavonoids, 6 carboxylic acids and derivatives, 6 isoflavonoids, 6 organooxygen compounds, and 2 prenol lipids. Twenty-seven characterized flavonoids were further assigned to flavonoid *O*-glycosides, flavonoid C-glycosides, *O*-methylated flavonoids, and flavonols.

For the discovery of primary metabolites, ^{1}H NMR has a good advantage. On the other hand, UPLC-MS results provided more abundant information on secondary metabolites. Therefore, based on the combination analysis, a total of 57 differential metabolites (10 of ^{1}H NMR and 47 of UPLC-MS) were screened and considered as differential biomarkers to distinguish hawthorn and insect-infested hawthorn samples.

3.5. Semi-quantitative analysis of differential biomarkers

To visualize the content variation of differential biomarkers, the heat map with HCA was applied. The red and blue color indicated higher and lower content of metabolite than the mean value, respectively (Senizza et al., 2019).

Based on 10 differential biomarkers from ^{1}H NMR analysis, the hawthorn, PI-hawthorn, and TC-hawthorn samples were separated and clustered into three categories (Fig. 5A), which was in accordance with

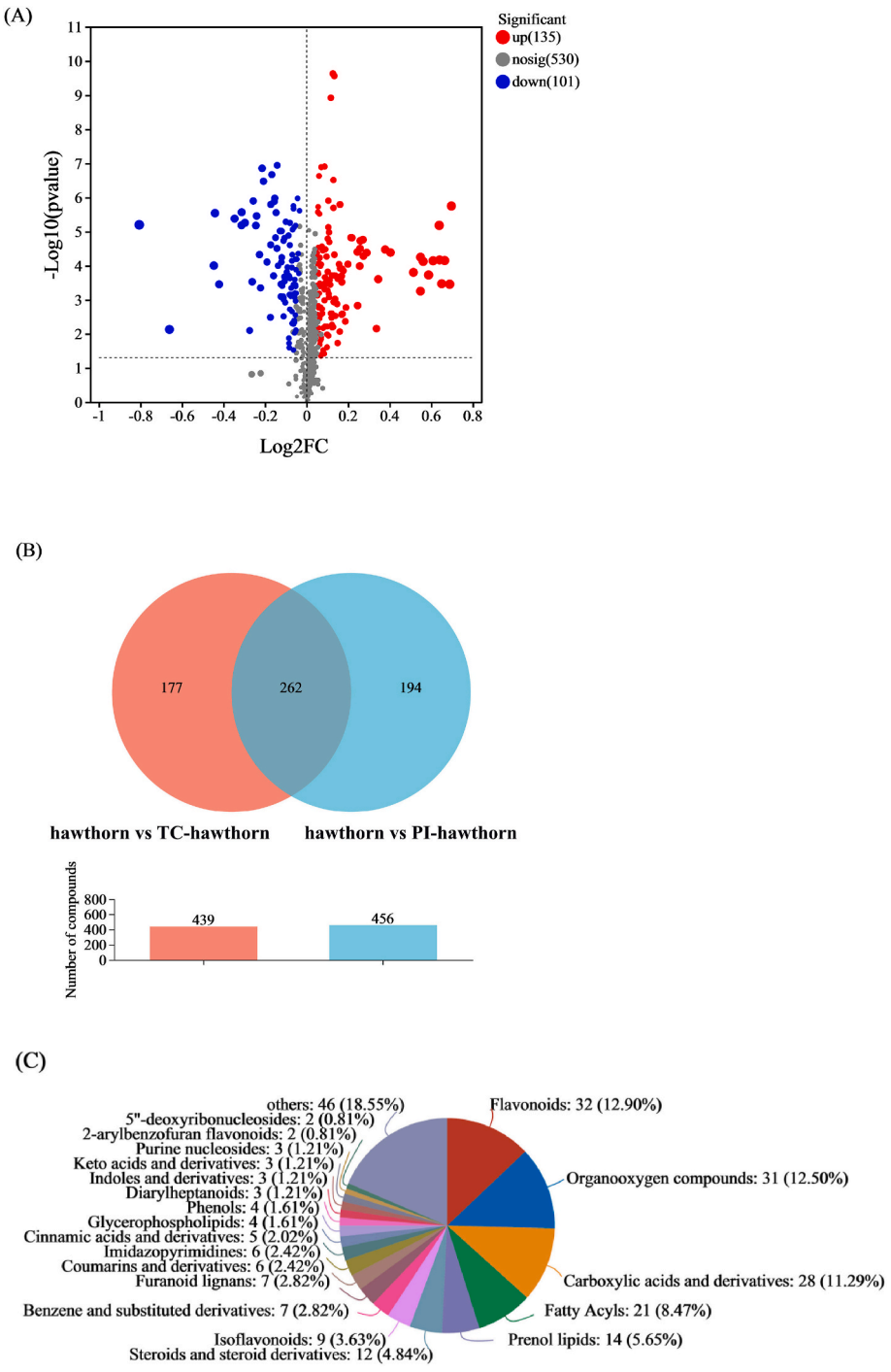

Fig. 4. Screening and analysis of differential metabolites based on UPLC-MS analysis. The volcano plot of hawthorn vs TC-hawthorn group in positive ion mode (A); Red and blue dots indicate up-regulated and down-regulated metabolites, respectively. Gray dots show metabolites without differences; The Venn diagram analysis of differential metabolites in comparison groups of hawthorn vs PI-hawthorn and hawthorn vs TC-hawthorn (B); Proportions of the 262 shared differential metabolites obtained by Venn diagram (C). PI-hawthorn: *P. interpunctella* infested hawthorn; TC-hawthorn: *T. castaneums* infested hawthorn. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the results of PCA and PLS-DA. As shown in Fig. 5A, the content of β-D-glucose, aspartic acid, citric acid, 3,4-*O*-dicaffeoylquinic acid, (−)-epigallocatechin, and quercetin was higher in hawthorn samples than that in the other two samples. It had been reported that insects needed a balanced proportion of carbohydrates, proteins, and fats for growth and development. So, substances rich in these nutrients were easily infested by insects (Cheng et al., 2023b). Therefore, the decreased content of β-D-glucose and aspartic acid might be absorbed and utilized by insects to maintain their own life activities in the process of insect infestation. As beneficial flavonoids and phenolic compounds in hawthorn, (−)-epigallocatechin, quercetin, and 3,4-*O*-dicaffeoylquinic acid had been reported to have significant antibacterial, anti-cancer, and antioxidant properties (Zhang et al., 2020; Zurek et al., 2021). As shown in Fig. 5A, their content in insect-infested hawthorn samples was lower than that in hawthorn samples, which could be inferred that antibacterial, anti-cancer, and antioxidant pharmacological activities decreased after insect-infestation.

The heat maps also were analyzed based on 47 screened differential

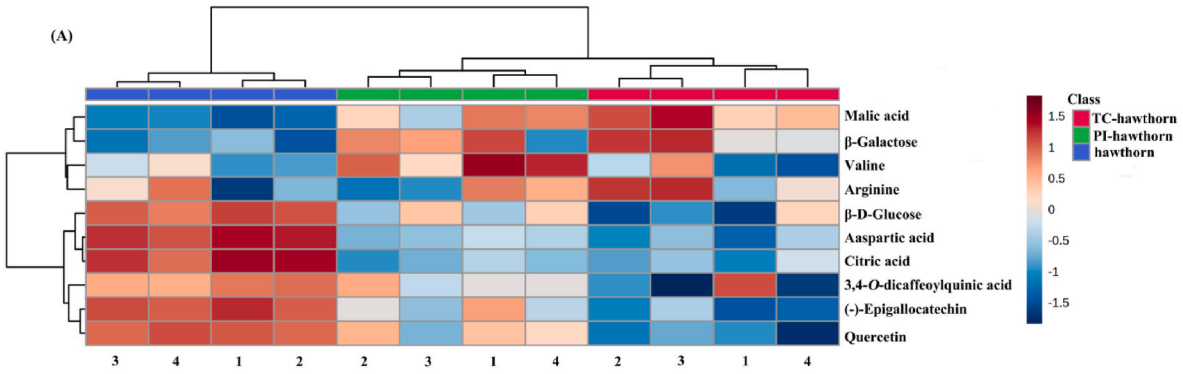

Fig. 5. Heat maps of differential biomarkers. Differential biomarkers screened from hawthorn, PI-hawthorn, and TC-hawthorn based on ^{1}H NMR analysis (A), from hawthorn and PI-hawthorn based on UPLC-MS analysis (B), and from hawthorn and TC-hawthorn based on UPLC-MS analysis (C). The red and blue color indicated higher and lower content of metabolite than the mean value, respectively. The number at the bottom of the spectra represents the four parallelism of each sample. PI-hawthorn: *P. interpunctella* infested hawthorn; TC-hawthorn: *T. castaneums* infested hawthorn. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

biomarkers of UPLC-MS analysis. As shown in Fig. 5B and C, the content of 21 differential biomarkers in insect-infested hawthorn samples was lower than that in hawthorn samples, including 11 flavonoids (4 flavonoid *O*-glycosides, 5 *O*-methylated flavonoids, and 2 flavonols), 5 carboxylic acids and derivatives, 2 isoflavonoids, 2 organooxygen compounds, and 1 prenol lipids. Among them, flavonoids were the most abundant metabolites. Previous studies had indicated that flavonoids were the key bio-active compounds in hawthorn with some activities of scavenging free radicals, promoting anti-oxidase or inhibiting oxidative enzymes, regulating the blood flow, and keeping the heart healthy (Shao et al., 2017; Wen et al., 2015). Astragalin, a flavonoid *O*-glycoside, had anti-oxidant, anti-inflammatory, anti-cancer, cardioprotective, neuro-protective, anti-obesity, anti-diabetic, anti-osteoporotic, and antiulcer properties (Riaz et al., 2018). Liquiritin, a flavonoid *O*-glycoside, had effects on anti-inflammatory, anti-depression, anti-Alzheimer's disease, liver protection, cardiovascular protection, anti-tumor, anti-tussive, and skin protection (Qin et al., 2022). Nobiletin, an *O*-methylated flavonoid, had properties on anti-inflammatory, anti-cancer, anti-diabetes, anti--atherosclerosis, and anti-obesity (Nakajima and Ohizumi, 2019). Dracorhodin, an *O*-methylated flavonoid, had anti-oxidant, anti-cancer, anti-microbial, anti-viral, and anti-tumor effects (Waluyo and Wibowo, 2018). 6-Methoxy-7-methylquercetin, a flavonol, had high anti-oxidant activity and high cytotoxic effect against MCF-7 and Hep G2 cells (Hussien et al., 2016). Tambulin, a flavonol, had anti-ageing, anti-oxidative, anti-diabetic, anti-cancer, and anti-Parkinsonian effects (Pandey et al., 2019; Wang et al., 2020). Based on these results, some activities of hawthorn, such as anti-oxidant, anti-inflammatory, and anti-tumor, could be weakened after insect-infestation.

In conclusion, these results of both ^{1}H NMR and UPLC-MS analysis indicated that there were significant differences on primary and secondary metabolites before and after insect infestation of hawthorn. Hawthorn is rich in various substances with health-promoting benefits, but their content decreased after insect infestation, which inevitably led to poor quality.

3.6. ROC curve of differential biomarkers

Differential biomarkers with high sensitivity and specificity are expected to be obtained and used to distinguish hawthorn samples and insect-infested hawthorn samples. In this context, a ROC curve and its area under curve (AUC) were applied to assess the distinguishing accuracy of screened differential biomarkers (Zhang et al., 2018). The coordinate point on the curve was the optimal threshold for distinguishing hawthorn and insect-infested hawthorn samples, along with the abscissa indicating specificity and the ordinate indicating sensitivity. Generally, the range of AUC value is 0.5–1.0, and the closer the value is to 1.0, the better the distinguishing accuracy is (Yan et al., 2020). In order to obtain more accurate differential biomarkers to distinguish hawthorn and insect-infested hawthorn samples, the high AUC value of 1 was adopted. As shown in Table 2, except to valine, arginine, 3, 4-*O*-Dicaffeoylquinic acid, β-Galactose, and Tambulin, the remaining 52 differential biomarkers (6 of ^{1}H NMR results and 46 of UPLC-MS results) had high sensitivity (1.0) and specificity (1.0). Based on above results, 52 differential biomarkers from ^{1}H NMR and UPLC-MS analysis had a powerful distinguishing performance for hawthorn and insect-infested hawthorn samples.

4. Conclusion

The combination analysis of ^{1}H NMR and LC-MS method was used to reveal complementary information on primary and secondary metabolites. These result could provide a systematic and comprehensive strategy to compare metabolite differences in hawthorn and insect-infested hawthorn (PI-hawthorn and TC-hawthorn) samples. Thirty-two metabolites, including amino acids, sugars, phenolic acids, and flavonoids, were identified by ^{1}H NMR analysis. A total of 1463 metabolites were identified by UPLC-MS analysis, including flavonoids, phenylpropanoids, amino acids related compounds, fatty acids related compounds, alkaloids, and terpenoids. Among them, flavonoids were the most abundant compounds. The spectra of both ^{1}H NMR and UPLC-MS showed that metabolite differences before and after insect-infestation of hawthorn were displayed in the content, rather than in the level of appearing or disappearing. According to the screening parameters of ^{1}H NMR with VIP >1 and $P < 0.05$ and UPLC-MS with VIP >1.0, $P < 0.05$, and (FC) > 1 or < 1, a total of 57 differential metabolites (10 of ^{1}H NMR analysis and 47 of UPLC-MS analysis) were considered as differential biomarkers. The heat map analysis indicated that the content of some differential biomarkers with significant pharmacological activities decreased after being insect-infested, which inevitably led to poor quality of hawthorn. Through ROC curve assessment, 52 differential biomarkers with high sensitivity and specificity (6 of ^{1}H NMR analysis and 46 of UPLC-MS analysis) could ultimately be used to distinguish whether insect infestation occurred in hawthorn. In conclusion, These study's results contribute to evaluate quality of hawthorn and ensure food safety for consumers. It also provides a foundation for research on the hawthorn infestation mechanism. On this basis, we can further explore the relevant genes that regulate the changes of differential metabolites through omics technology to enrich the content of hawthorn infestation mechanism. In addition, we can also design a sensor to monitor the security of storage environment through the changes of

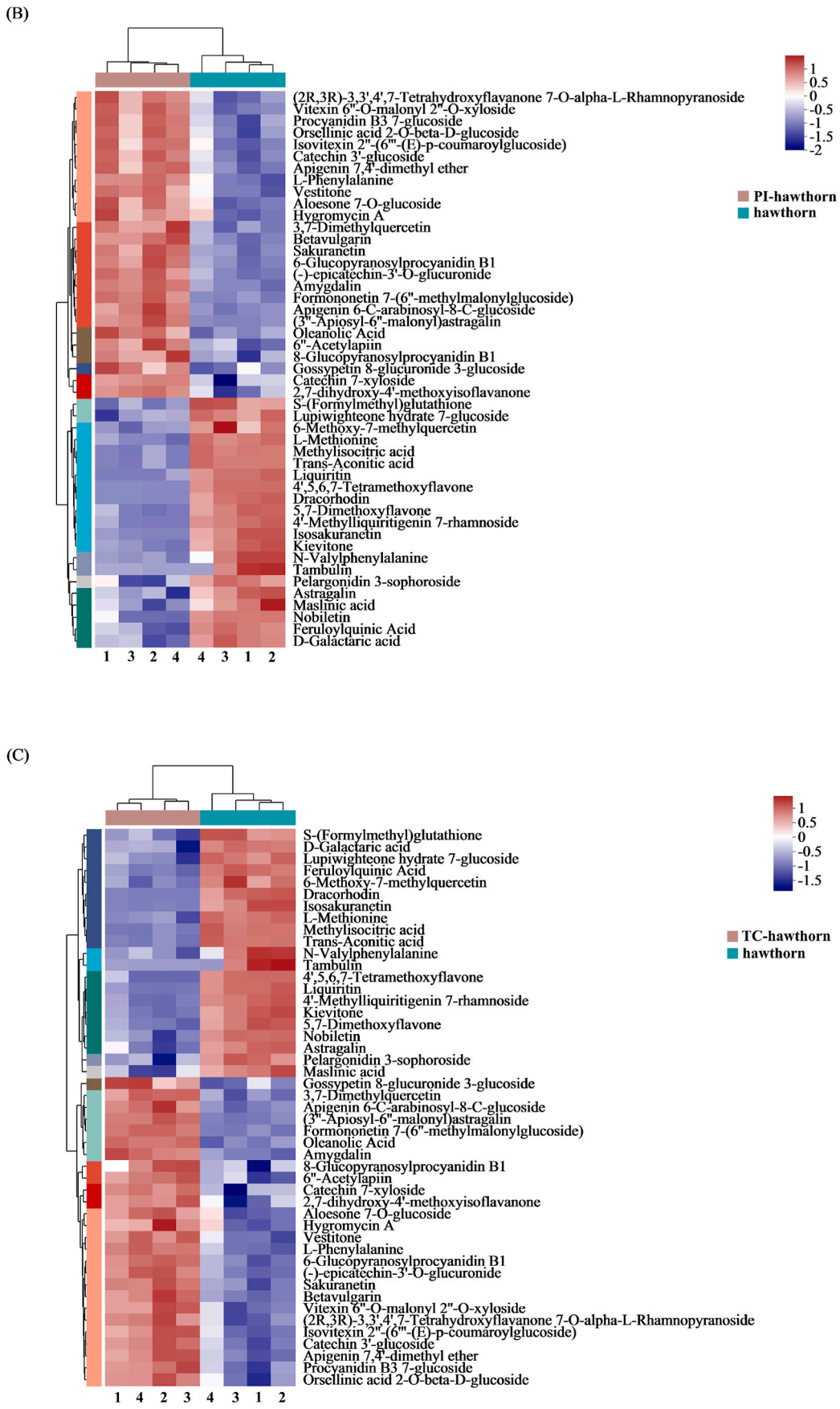

Fig. 5. (*continued*).

Table 2
The distinguishing accuracy of differential biomarkers based on ROC curve.

No.	Metabolites	Hawthorn *vs* PI-hawthorn[b]			Hawthorn *vs* TC-hawthorn[b]		
		AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
1	Valine [a]	1.0	1.0	1.0	0.63	1.0	0.5
2	(−)-Epigallocatechin	1.0	1.0	1.0	1.0	1.0	1.0
3	Aspartic acid	1.0	1.0	1.0	1.0	1.0	1.0
4	Citric acid	1.0	1.0	1.0	1.0	1.0	1.0
5	β-D-Glucose	1.0	1.0	1.0	1.0	1.0	1.0
6	Arginine [a]	0.5	0.5	0.8	0.75	1.0	0.5
7	Quercetin	1.0	1.0	1.0	1.0	1.0	1.0
8	3,4-*O*-Dicaffeoylquinic acid [a]	0.86	1.0	0.8	0.75	1.0	0.8
9	Malic acid	1.0	1.0	1.0	1.0	1.0	1.0
10	β-Galactose [a]	0.86	1.0	0.8	1.0	1.0	1.0
11	Hygromycin A	1.0	1.0	1.0	1.0	1.0	1.0
12	6″-Acetylapiin	1.0	1.0	1.0	1.0	1.0	1.0
13	(3″-Apiosyl-6″-malonyl) astragalin	1.0	1.0	1.0	1.0	1.0	1.0
14	Methylisocitric acid	1.0	1.0	1.0	1.0	1.0	1.0
15	S-(Formylmethyl) glutathione	1.0	1.0	1.0	1.0	1.0	1.0
16	2,7-dihydroxy-4′-methoxyisoflavanone	1.0	1.0	1.0	1.0	1.0	1.0
17	6-Glucopyranosylprocyanidin B1	1.0	1.0	1.0	1.0	1.0	1.0
18	Astragalin	1.0	1.0	1.0	1.0	1.0	1.0
19	Lupiwighteone hydrate 7-glucoside	1.0	1.0	1.0	1.0	1.0	1.0
20	Pelargonidin 3-sophoroside	1.0	1.0	1.0	1.0	1.0	1.0
21	(2R,3R)-3,3′,4′,7-Tetrahydroxyflavanone 7-*O*-alpha-L-rhamnopyranoside	1.0	1.0	1.0	1.0	1.0	1.0
22	Apigenin 6-C-arabinosyl-8-C-glucoside	1.0	1.0	1.0	1.0	1.0	1.0
23	D-Galactaric acid	1.0	1.0	1.0	1.0	1.0	1.0
24	Oleanolic acid	1.0	1.0	1.0	1.0	1.0	1.0
25	Catechin 3′-glucoside	1.0	1.0	1.0	1.0	1.0	1.0
26	Dracorhodin	1.0	1.0	1.0	1.0	1.0	1.0
27	4′,5,6,7-Tetramethoxyflavone	1.0	1.0	1.0	1.0	1.0	1.0
28	Procyanidin B3 7-glucoside	1.0	1.0	1.0	1.0	1.0	1.0
29	L-Methionine	1.0	1.0	1.0	1.0	1.0	1.0
30	3,7-Dimethylquercetin	1.0	1.0	1.0	1.0	1.0	1.0
31	N-Valylphenylalanine	1.0	1.0	1.0	1.0	1.0	1.0
32	Apigenin 7,4′-dimethyl ether	1.0	1.0	1.0	1.0	1.0	1.0
33	Catechin 7-xyloside	1.0	1.0	1.0	1.0	1.0	1.0
34	Liquiritin	1.0	1.0	1.0	1.0	1.0	1.0
35	Isovitexin 2′'-(6‴-(E)-*p*-coumaroylglucoside)	1.0	1.0	1.0	1.0	1.0	1.0
36	Sakuranetin	1.0	1.0	1.0	1.0	1.0	1.0
37	Amygdalin	1.0	1.0	1.0	1.0	1.0	1.0
38	6-Methoxy-7-methylquercetin	1.0	1.0	1.0	1.0	1.0	1.0
39	trans-Aconitic acid	1.0	1.0	1.0	1.0	1.0	1.0
40	Formononetin 7-(6″-methylmalonylglucoside)	1.0	1.0	1.0	1.0	1.0	1.0
41	4′-Methylliquiritigenin 7-rhamnoside	1.0	1.0	1.0	1.0	1.0	1.0
42	L-Phenylalanine	1.0	1.0	1.0	1.0	1.0	1.0
43	5,7-Dimethoxyflavone	1.0	1.0	1.0	1.0	1.0	1.0
44	Nobiletin	1.0	1.0	1.0	1.0	1.0	1.0
45	Kievitone	1.0	1.0	1.0	1.0	1.0	1.0
46	Feruloylquinic acid	1.0	1.0	1.0	1.0	1.0	1.0
47	Orsellinic acid 2-*O*-β-D-glucoside	1.0	1.0	1.0	1.0	1.0	1.0
48	(−)-epicatechin-3′-*O*-glucuronide	1.0	1.0	1.0	1.0	1.0	1.0
49	Vestitone	1.0	1.0	1.0	1.0	1.0	1.0
50	Gossypetin 8-glucuronide 3-glucoside	1.0	1.0	1.0	1.0	1.0	1.0
51	Tambulin [a]	0.81	1.0	0.75	0.75	1.0	0.75
52	Betavulgarin	1.0	1.0	1.0	1.0	1.0	1.0
53	Isosakuranetin	1.0	1.0	1.0	1.0	1.0	1.0
54	Maslinic acid	1.0	1.0	1.0	1.0	1.0	1.0
55	Vitexin 6″-*O*-malonyl 2″-*O*-xyloside	1.0	1.0	1.0	1.0	1.0	1.0
56	8-Glucopyranosylprocyanidin B1	1.0	1.0	1.0	1.0	1.0	1.0
57	Aloesone 7-*O*-glucoside	1.0	1.0	1.0	1.0	1.0	1.0

[a] Represents the AUC values of differential biomarkers was less than 1.
[b] PI-hawthorn represents *P. interpunctella* infested hawthorn; TC-hawthorn represents *T. castaneums* infested hawthorn.

differential metabolites using computer technology.

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CRediT authorship contribution statement

Yunxia Cheng: Conceptualization, Data curation, Methodology, Software, Writing – original draft. **Zhenying Liu:** Investigation, Software, Supervision, Visualization. **Bo Xu:** Software, Supervision. **Pingping Song:** Investigation, Visualization. **Zhimao Chao:** Resources, Funding acquisition, Project administration, Conceptualization, Writing - review & editing.

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100616.

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