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Characterization of a distinctive marker for monofloral honey of *Bauhinia championii* (Benth.) Benth. by combining untargeted, and targeted mass spectrometry analyses

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ABSTRACT

The monofloral honey derived from *Bauhinia championii* (Benth.) Benth. (MH-*Bc*) possesses significant nutritional and bioactive value, making it highly suitable for commercial exploitation. However, the poorly defined characteristics and unknown composition have hindered MH-*Bc* product development. In this study, we employed a combination of untargeted and targeted mass spectrometry analyses to characterize MH-*Bc* honey. As a result, 4,7,8-trimethoxydibenzo[b,d]furan-3-ol (TDBF) was identified as a robust chemical marker for distinguishing MH-*Bc* from other types of honey. This specific marker was detected in both MH-*Bc* and the *Bc* plant but was absent in other honey varieties. Furthermore, a targeted mass spectrometry quantitative method was developed and validated to accurately determine the content of TDBF in honey samples. Overall, the presence of TDBF serves as a discerning indicator for future commercial MH-*Bc* products.

1. Introduction

Bauhinia championii (Benth.) Benth. (*Bc*), a member of the Leguminosae family, exhibits exceptional efficacy in enhancing blood circulation and alleviating pain[1]. It has been utilized in functional food or traditional medicine for the management of rheumatoid arthritis, acute and chronic back and leg pain, as well as epigastric discomfort[2]. Many studies have shown that *Bc* contains a wealth of bioactive compounds capable of combating cancer, preventing acute ischemic injury, and regulating cell apoptosis[3]. Consequently, it is hypothesized that the monofloral honey of *Bc* (MH-*Bc*) may contain similar bioactive constituents with potential therapeutic benefits. However, there are currently no standardized methods for determining MH-*Bc* composition. The preservation of consumer interests and the promotion of sustainable development in the food industry necessitates the development and implementation of a distinguishing technique to accurately assess the characteristics and authenticity of MH-*Bc*.

Numerous studies have not only determined the composition of honey, but also examined the significant impact of the botanical and geographical origin of honey on its composition[4-6]. For instance, the

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active ingredients that distinguish Manuka honey from other types of honey are kojic acid, 5-methyl-3-furancarboxylic acid, leptosin, unedone, 2-methoxybenzoic acid, etc.[6]. Monofloral honey derived from the nectar of traditional Chinese medicinal plants as a source of high-value chemicals has been a focal point of our research in recent years. We have developed a mass spectrometry technique for the detection of specific markers in *Carthamus tinctorius* L. (safflower) monofloral honey (MH-*Ct*), *Astragalus membranaceus* var. *mongholicus* Hsiao monofloral honey (MH-*Am*), and *Lespedeza bicolor* Turcz. monofloral honey (MH-*Lb*). Safflomin A has been identified as a unique marker for the MH-*Ct*[7]. The chemical markers, calycosin and formononetin, are reliable indicators for the accurate identification of MH-*Am*[8]. Furthermore, it was confirmed that kaempferol-3-*O*-galactoside serves as the distinctive marker compound for MH-*Lb*[9].

Based on our research platform, we aimed to develop a method for identifying MH-*Bc* based on its unique chemotypes. Thus, we conducted a comparative analysis between MH-*Bc* and various types of honey using untargeted mass spectrometric approaches to identify distinctive markers associated with MH-*Bc*. The presence and abundance of a specific marker in MH-*Bc*, other types of honey, as well as *Bc* plants, were determined using our newly established targeted mass spectrometry technique. Taken together, our findings support future quality assurance and authenticity standards of commercial MH-*Bc* products.

2. Materials and methods

2.1 Chemicals and reagents

HPLC-grade acetonitrile, methanol, ethanol, and formic acid were purchased from Fisher Scientific Inc. (Pittsburgh, USA). Syringe filters (Nylon, 0.22 μm) and the QuEChERs extract pouch (EN Method 5992-0650) were purchased from Agilent (Santa Clara, USA). HPLC-grade standards of 4,7,8-trimethoxydibenzo[b,d] furan-3-ol (TDBF), with a purity of 97.3% (Fig. S1 and Table S1), was synthesized at WuXi AppTec Co., Ltd. (Jiangsu, China). Standard stock solutions of TDBF at a concentration of 1 mg/mL were prepared in methanol and stored at –20 ℃. The stock solution was diluted to different concentration standard solutions and stored at 4 ℃ until further analysis.

2.2 Sample collection

A total of 70 monofloral honey samples including MH-*Bc*, *Vitex negundo* var. *heterophylla* (Franch.) Rehd. honey (MH-*Vn*), *Robinia pseudoacacia* L. honey (MH-*Rp*), *Ziziphus jujuba* Mill. honey (MH-*Zj*), and MH-*Am* were collected from representative apiaries in China by skilled beekeepers and researchers. These apiaries had sizable monofloral areas that covered the bees' collection range during the flowering season to ensure sample authenticity for this study. Among them, the MH-*Bc* and MH-*Am* honey samples were collected by *Apis cerana* honeybees. The MH-*Vn*, MH-*Rp*, and MH-*Zj* honey samples were collected by *Apis mellifera* honeybees. The MH-*Vn*, MH-*Rp*, and MH-*Zj* honey samples were used for comparison with MH-*Bc* to identify the distinguishing component in MH-*Bc*. The MH-*Am* honey samples were used to validate the accuracy of the identified compound for distinguishing MH-*Bc* honey. Details of the samples are listed in Table S2. Palynological analysis was executed in line with the protocol outlined by Zhao et al.[7]. It revealed that approximately 60% of pollen grains in MH-*Bc* samples were consistent with the pollen from *Bc* blossoms, confirming that these samples meet the required threshold (> 45%) for classification as genuine monofloral honey[10]. After collection, honey samples were maintained freshness by storing at 4 ℃, and then extracted to conduct detailed experiments within a month. The honey samples are kept in their natural state and have not undergone any other processing.

2.3 Determination of physicochemical properties of MH-Bc

The physicochemical properties of representative samples were assessed and their respective thresholds were established. The moisture content, pH level, amylase activity, as well as glucose, fructose, and sucrose contents of the MH-*Bc* samples were evaluated using the AOAC method[11]. The concentrations of 5-hydroxymethylfurfural (5-HMF) and 3-deoxyglucose aldehyde ketone (3-DG) were analyzed as indicators for assessing the freshness of honey samples[12-13]. The total phenolic compounds and flavonoids were quantified using the Folin-Ciocalteu and aluminum nitrate colorimetric methods, respectively[14-15].

2.4 Sample preparation

The QuEChERS method for extraction and purification was optimized based on previously reported procedures[16]. We accurately measured (5.00 ± 0.01) g of honey in a centrifuge tube, followed by the addition of 5 mL of water. Contents were gently swirled for 2 min. Subsequently, 10 mL of acetonitrile was added to the mixture and vortexed for 2 min. After incorporating the QuEChERs Extract pouch (EN Method5992-0650), the mixture was vortexed for another 2 min. The supernatant was obtained by centrifuging at a speed of 6 000 × *g* for 10 min at 4 ℃. An equivalent volume (approximately 8 mL) of supernatant was collected from each sample and evaporated under vacuum conditions at a rotation speed of 600 × *g* in a temperature range between 35–40 ℃. Finally, the dried extracts were dissolved in 1 mL of methanol, followed by filtration through a nylon filter membrane with a pore size of 0.22 μm.

2.5 Non-targeted analysis by ultra-high performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) technology

An UPLC-Q-TOF-MS/MS system (Agilent, CA, USA) was used to identify chemical markers from samples. The injection volume was 2 μL. A ZORBAX Eclipse Plus C_{18} column (2.1 mm × 100 mm, 1.8 μm) was used at a temperature of 50 ℃. Mobile phase A was ultrapure water with 0.1% (*V*/*V*) formic acid, and mobile phase B was acetonitrile with 0.1% (*V*/*V*) formic acid, respectively. The flow rate was set to 0.3 mL/min. The gradient elution program was set as follows: 0 min, 5% B; 2 min, 5% B; 20 min, 100% B; 25 min, 100% B; post time, 5 min. The MS parameters were set as follows: drying gas temperature, 325 ℃; drying gas flow rate, 10 L/min; sheath gas

temperature, 370 ℃; sheath gas flow rate, 12 L/min; nebulizer pressure, 35 psi; V Cap, 3 500 V; fragmenter voltage, 135 V; mass ranges, *m*/*z* 100–1 700, acquired in positive ionization mode. The Agilent Profinder B.08.00 software executed various techniques, including mass molecular feature extraction, peak alignment, and peak area integration. Agilent IDBrowser B.08.00 and Mass Profiler Professional software 15.0 were used for the identification of compounds by matching the METLIN database (DB) with a threshold of mass errors < 5×10^{-6} (0.000 5%) and DB score > 80.

2.6 Targeted analysis by UPLC-QqQ-MS/MS technology

2.6.1 Instrument method

An Agilent ultra-high performance liquid chromatography with triple quadrupole tandem mass spectrometry (UPLC-QqQ-MS/MS) was used to conduct quantitative analysis on the identified markers. A binary solvent system was used, in which mobile phase A consisted of ultrapure water with 0.1% (*V*/*V*) formic acid, and mobile phase B consisted of acetonitrile with 0.1% (*V*/*V*) formic acid. The flow rate was 0.3 mL/min and the injection volume was 2 μL. The gradient elution program for each run started at 10% B and was kept at 10% B for 1 min, then increased to 95% B at 2 min, and was kept at 95% B from 2 to 6 min, then decreased to 10% B at 6.1 min, and was kept at 10% B from 6.1 to 7 min. Post time was 1 min. Eluents from 0 to 1 min were diverted to waste. For sample separation, an Agilent Eclipse Plus C_{18} RRHD column (2.1 mm × 50 mm, 1.8 μm) was used and the column was maintained at 40 ℃.

For MS analysis, mass spectral data acquisition was achieved using the multiple reaction monitoring (MRM) mode, acquired in positive ionization mode. The electrospray ionization (ESI) settings were as follows: dry gas temperature, 200 ℃; dry gas flow rate, 15 L/min; nebulizer pressure, 45 psi; sheath gas temperature, 330 ℃; sheath gas flow rate, 12 L/min; nozzle voltage, 1.5 kV; capillary voltage, 3.5 kV. The mass spectral information of targeted compounds and MRM transitions are detailed in Table 1. Standard calibration curves were constructed based on the optimized quantitative ion pairs.

2.6.2 Method validation

To validate the accuracy and reliability of the results, quantitative methods were validated by linearity, limit of detection (LOD), limit of quantitation (LOQ), recovery rate, matrix effect, and precision. We displayed the peak area of 6 concentrations of labeled standard matter (2–300 μg/L, estimated as concentrations in honey), resulting in matrix-matched calibration curves to assess linearity and coefficient of determination (R^2). The LOD and LOQ were determined when the signal-to-noise (S/N) ratio reached approximately 3 and 10, respectively. The recovery rate was evaluated after the blank honey samples were spiked at three concentration levels of 2, 4 and 40 μg/kg each. The term "blank honey samples" refers to those kinds of honey samples that do not contain the intended analytes. The slope ratio between the solvent-based calibration curve and the matrix-matched calibration curve was used to investigate matrix effects. The relative standard deviation (RSD), which serves as an indicator of method accuracy, was calculated based on 6 replicates for each sample at peak values of 2, 4 and 40 μg/kg.

2.7 Statistical analysis

For statistical analysis, data sets were loaded into the SIMCA 14.1 program (Sartorius Stedim Biotech Ltd., Umea, Sweden) for principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). One-way analysis of variance (ANOVA) was used to test the significance of differences in chemical marker levels among different types of honey using SPSS version 26.0 software. The significance level was set at P value < 0.05. Data of all measurements were expressed as mean ± standard deviation.

3. Results and discussion

3.1 The determination of physicochemical properties of MH-Bc

The assessment of honey quality heavily relies on physicochemical indexes. The evaluated parameters in MH-*Bc* encompass moisture content, pH value, amylase activity, levels of 5-HMF and 3-DG, quantities of total phenolic compounds and flavonoids, as well as the soluble sugar contents of glucose, fructose, and sucrose.

The moisture content of honey serves as an important indicator for assessing its maturity. According to international standards, the moisture level of honey should not exceed 20%[17]. The measurement results indicate that the moisture content of MH-*Bc* ranged from 16.77 to 21.70 g water per 100 g honey, with an average value of 19.14 g/100 g (Table 2). According to previous publications, the moisture content of mature honey typically ranges from 13.8% to 18.7%, which is influenced by various factors such as climate, botanical origin, geographical location, and bee species[18-19]. Due to the high humidity of the production area of MH-*Bc* (Southern China), and the absence of well-established standards for MH-*Bc* production, a minor proportion of samples exhibit a slightly higher moisture content exceeding the standard of 20%. Therefore, it is imperative to establish standardized production protocols for mature MH-*Bc* to effectively regulate product quality.

Table 1
Specific parameters for the quantification of TDBF by UPLC-QqQ-MS/MS.

Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Fragmentor voltage (V)	Collision energy (V)	Linear range (μg/L)	R^2	LOD (μg/kg)	LOQ (μg/kg)
	200		20				
275	243*	120	20	2–300	0.999 6	0.5	2
	260		30				

Note: *Product ion for quantitation.

Table 2
The results of physicochemical indexes detected in MH-*Bc* samples.

Sample	Moisture content (g/100 g)	pH	Amylase value (mL/(g·h))	5-HMF (mg/kg)	3-DG (mg/kg)	Sucrose (g/100 g)	Fructose (g/100 g)	Glucose (g/100 g)	Total phenols (mg GAE/kg)	Total flavonoids (mg RE/kg)
1	21.70 ± 1.41	3.69 ± 0.20	18.53 ± 3.48	1.25 ± 0.48	99.59 ± 14.73	0.51 ± 0.71	39.98 ± 4.51	33.11 ± 2.84	298.30 ± 16.87	9.35 ± 2.76
2	17.22 ± 1.25	3.60 ± 0.04	22.04 ± 4.30	2.59 ± 1.01	88.17 ± 15.03	ND	40.35 ± 6.11	33.11 ± 3.30	238.44 ± 40.69	9.99 ± 1.48
3	19.18 ± 1.52	3.81 ± 0.11	19.05 ± 4.27	3.26 ± 1.15	123.65 ± 15.87	ND	45.50 ± 1.77	31.99 ± 4.26	335.70 ± 43.85	10.60 ± 1.70
4	18.80 ± 4.95	3.84 ± 0.07	17.03 ± 2.86	2.44 ± 0.91	76.28 ± 25.06	ND	45.47 ± 4.60	31.89 ± 5.01	344.21 ± 36.95	9.98 ± 1.05
5	20.20 ± 2.12	3.85 ± 0.11	17.04 ± 2.88	2.03 ± 0.40	96.45 ± 18.79	0.88 ± 0.08	41.08 ± 3.32	29.80 ± 0.83	360.72 ± 35.95	10.72 ± 0.82
6	19.20 ± 2.83	3.90 ± 0.15	22.95 ± 7.33	2.43 ± 0.76	111.84 ± 12.30	1.18 ± 1.67	43.47 ± 2.41	28.01 ± 2.14	320.28 ± 41.13	10.03 ± 1.36
7	18.15 ± 1.48	3.73 ± 0.08	19.45 ± 4.17	2.42 ± 1.01	106.57 ± 5.90	0.51 ± 0.72	41.07 ± 3.83	26.90 ± 0.13	353.65 ± 24.01	12.30 ± 4.33
8	19.00 ± 0.42	3.77 ± 0.14	23.51 ± 6.72	2.81 ± 1.46	105.30 ± 12.78	ND	40.93 ± 3.05	29.26 ± 2.78	307.72 ± 22.49	13.98 ± 5.77
9	17.70 ± 2.83	3.86 ± 0.11	19.05 ± 5.01	2.85 ± 1.27	132.07 ± 20.12	ND	40.29 ± 3.65	27.66 ± 1.42	305.60 ± 41.14	12.91 ± 3.46
10	20.70 ± 0.71	3.76 ± 0.06	23.12 ± 7.05	1.95 ± 0.72	82.06 ± 17.67	1.06 ± 1.50	41.98 ± 0.89	30.06 ± 0.32	283.22 ± 26.67	12.59 ± 2.52
11	18.43 ± 3.15	3.75 ± 0.14	27.10 ± 7.13	4.39 ± 0.81	130.86 ± 9.72	0.50 ± 0.70	39.83 ± 1.59	29.41 ± 0.30	335.47 ± 64.30	15.53 ± 1.87
12	19.20 ± 2.83	3.88 ± 0.15	23.88 ± 2.03	2.26 ± 0.98	113.17 ± 12.73	ND	42.79 ± 4.14	31.53 ± 0.80	273.97 ± 47.86	11.09 ± 3.46
13	19.45 ± 1.77	3.79 ± 0.13	20.03 ± 7.11	1.65 ± 0.72	117.61 ± 22.44	1.29 ± 1.82	42.87 ± 0.53	30.50 ± 1.00	320.65 ± 26.26	15.80 ± 4.72
14	16.77 ± 2.51	3.87 ± 0.11	23.71 ± 7.13	3.28 ± 1.70	149.50 ± 19.80	1.73 ± 0.57	41.14 ± 3.22	31.87 ± 4.75	328.05 ± 49.54	23.90 ± 14.76
15	21.53 ± 6.90	3.81 ± 0.14	25.16 ± 9.93	2.59 ± 1.92	109.91 ± 15.51	ND	40.11 ± 0.87	29.25 ± 1.72	324.98 ± 39.65	14.06 ± 6.36
Average	19.14 ± 2.53	3.79 ± 0.12	22.95 ± 7.33	2.54 ± 1.08	109.53 ± 22.78	0.51 ± 0.83	41.79 ± 3.03	30.29 ± 2.69	315.40 ± 42.35	12.85 ± 5.13

Note: GAE, gallic acid equivalent; RE, rutin equivalent. Values are mean ± standard deviation (n = 2, between MH-*Bc* collected in 2021 and 2022 from the same apiarium). ND means not detected.

The pH level of honey is indicative of its stability and shelf life. In the majority of cases, honey is acidic and has a pH value between 3.2 and 4.5, which allows it to prevent the development of a number of bacterial infections[20]. For MH-*Bc*, the pH value of samples ranged from 3.6 to 3.9, with a mean of 3.79 (Table 2).

The freshness of honey can be assessed based on its amylase activity, which is indicative of the degree of sugar breakdown during the brewing process. Usually, high amylase values in honey indicate that the honey is a raw or minimally processed honey, which has not been heated to high temperatures. Raw or minimally processed honey has a higher nutritional value and a more intense flavor than processed honey. Therefore, a high amylase value can reflect the economic value of honey by suggesting that it is a high-quality, natural product that is more expensive to produce. Typically, lower α-amylase activity is observed in expired honey. Key glycolytic enzymes found in honey include amylase, sucrase, glucose oxidase, and glucosidase[21]. The amylase values of MH-*Bc* samples ranged from 17.03 to 27.10 mL/(g·h) (Table 2). This outcome significantly exceeds the international standards which require a minimum amylase value of no less than 3 mL/(g·h)[17]. The high amylase activity also reflects the high biological activity, freshness, and economic value of MH-*Bc*.

Upon exposure to heat, honey undergoes the Maillard reaction, leading to the formation of hydroxymethylfurfural (HMF)[22]. In addition, storage conditions and duration also influence the formation of HMF, making it a reliable indicator for assessing honey quality[23]. Annex II of the Council Directive 2001/110/EC established composition criteria for HMF levels in honey[24]. The permissible limit for HMF was set at 40 mg/kg, except for baker honey. For honey declared as originating from a tropical region, the maximum limit for HMF is raised to 80 mg/kg. The sample analysis findings indicate the presence of detectable 5-HMF in MH-*Bc* samples, ranging from 1.25 to 4.39 mg/kg (Table 2), which readily meets the requirements mentioned above. The presence of 3-DG can also serve as an indicator for evaluating the extent of honey overheating because 3-DG plays a crucial role as an intermediate in the Maillard reaction[25]. The results revealed that the content of 3-DG ranged from 76.28 to 149.50 mg/kg (Table 2), which is far less than the concentration of 3-DG in previously observed honeys which ranged from 187 to 884 mg/kg[25-26].

Moreover, the combined amount of glucose and fructose in MH-*Bc* ranged from 64.36 to 77.95 g/100 g honey, and the amount of sucrose was no more than 3 g/100 g (Table 2), meeting the criteria of a minimum of 60 g/100 g of fructose and glucose (sum of both) and a maximum of 5 g/100 g of sucrose[17]. Previously study indicated that honey has a fructose concentration ranging from 36.03 to 48.72 g/100 g and a fructose/glucose ratio ranging from 0.4 to 1.6 or even higher[27-28]. The variations in sugar contents primarily stem from differences in floral origin, geographical origin, and environmental factors.

Polyphenols are the main active components of honey, derived from plant secondary metabolism. In recent years, extensive research has been conducted on phenols and flavonoids, revealing their significant bioactivities in honey[29]. Similar to sugar content and composition, different botanical sources confer different total phenolic content (TPC) and total flavonoid content (TFC) in kinds of honey. Acacia honey displays a TPC range between 36.6 to 52.7 mg GAE/kg and a TFC range of 9.14 to 12.17 mg RE/kg[30-31]. Chestnut honey exhibits a TPC range of 12.92 to 212.7 mg GAE/kg[32]. Jujube honey has a TPC level ranged from 114.63 to 190.79 mg GAE/kg, and TFC level with a range of 3.40 to 16.43 mg RE/kg[31,33-34]. Additionally, we detected that TPC and TFC in MH-*Vn* ranged 330.24–520.62 mg GAE/kg

and 19.05–31.19 mg RE/kg; TPC and TFC in MH-*Rp* ranged 25.81–76.63 mg GAE/kg and 8.09–15.22 mg RE/kg; TPC and TFC in MH-*Zj* ranged 98.09–194.79 mg GAE/kg and 4.87–18.98 mg RE/kg; TPC and TFC in MH-*Am* ranges 87.85–163.03 mg GAE/kg and 7.95–18.56 mg RE/kg (Table S3). Comparing with other various honeys, we found that MH-*Bc* exhibits TPC ranging from 238.44 to 360.72 mg GAE/kg, and TFC ranging from 9.35 to 23.90 mg RE/kg (Table 2).

3.2 Untargeted analysis for MH-Bc markers screening based on UPLC-Q-TOF-MS/MS

Previous decades of research have established a wide range of techniques for validating the characteristics and composition of honey. The fundamental approach for honey characterization involves conducting a comprehensive analysis of its physical and chemical properties, as outlined in Section 3.1. Another traditional method for identifying the botanical origin of honey is through palynology detection, as the diverse plant species produce a wide range of honey types, each containing distinct pollen species[35]. However, inadvertent incorporation of pollen from diverse flora species during honey collection, processing, and transit may lead to potential inaccuracies and variation in analysis. Therefore, in this study, we aimed to develop a more precise identification approach by employing novel methods to distinguish MH-*Bc* from other types of honey. Distinctive indicators for MH-*Bc* were identified using a non-targeted metabolomic and chemometric approach.

The composition and relative content of compounds in 30 MH-*Bc* samples, 10 MH-*Vn* samples, 10 MH-*Rp* samples, 10 MH-*Zj* samples, and 5 *Bc* plant samples (Table S2) were analyzed using UPLC-Q-TOF-MS/MS to investigate the characteristic substances present in MH-*Bc*. We identified 101 unique compounds present in both the plant

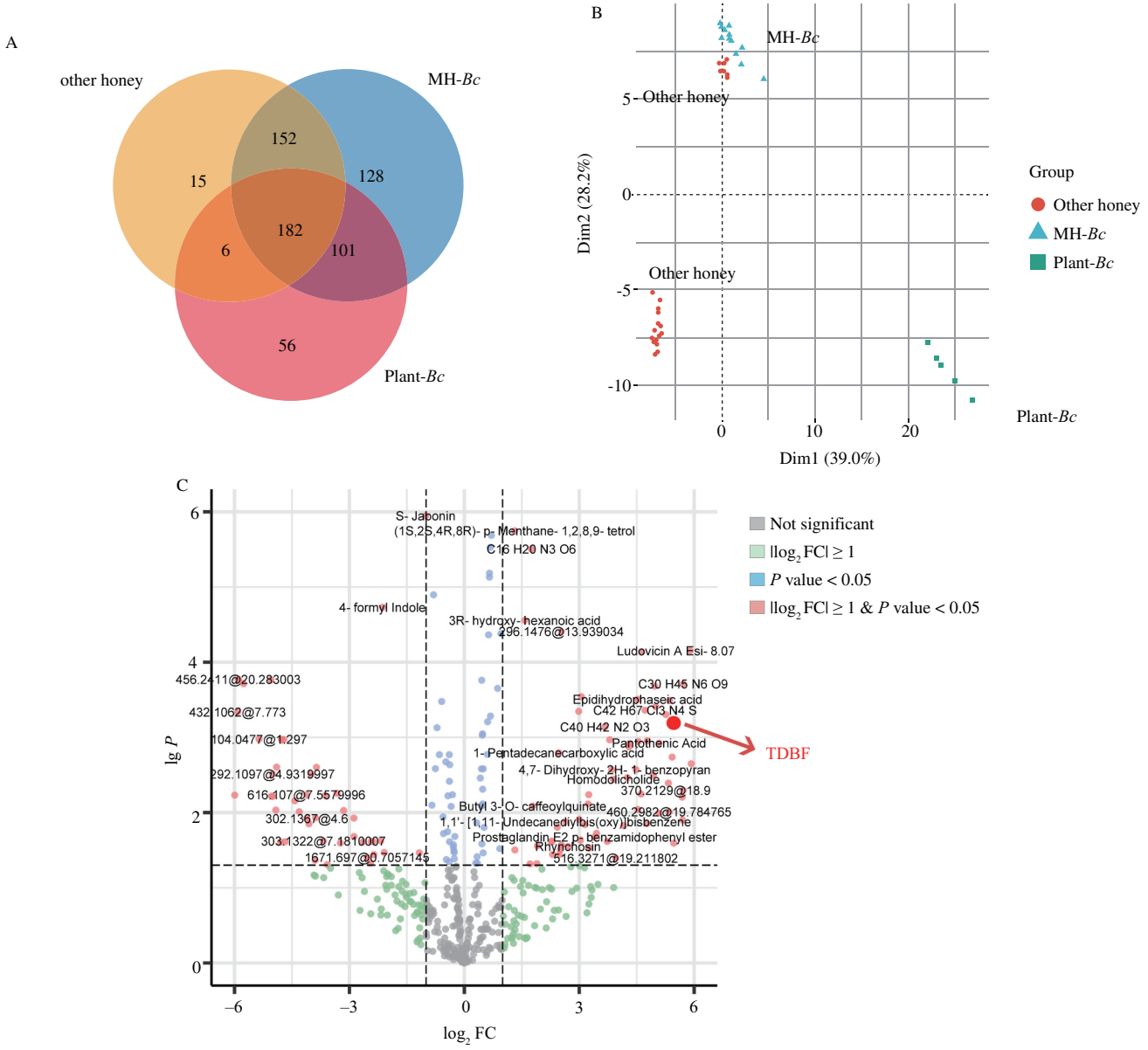

Fig. 1 UPLC-Q-TOF-MS/MS-based untargeted analysis among samples. (A) Venn diagram for comparison of compounds in monofloral honey samples of MH-*Bc*, other types of monofloral honey samples, and the plant samples of Plant-*Bc*. The Venn diagram is based on data from compounds with VIP > 1. (B) PCA among different groups of samples. (C) Volcano plot analysis between MH-*Bc* and other honey samples.

samples of *Bc* (plant-*Bc*) and MH-*Bc* but absent in the other honeys (Fig. 1A). PCA analysis was employed to visually represent the differences among MH-*Bc*, plant-*Bc*, and other honey samples, clearly demonstrating their distinct characteristics (Fig. 1B). Furthermore, a volcano plot analysis was conducted (P value < 0.05 and fold change (FC) > 2.0) to effectively discriminate the MH-*Bc* signatures (Fig. 1C). Additionally, a cluster heatmap analysis was performed to analyze the relative content of compounds in each sample (Fig. S2). Notably, there are significant differences between the composition of MH-*Bc* and that of other types of honey.

By employing the partial least squares regression methodology, we have developed a dynamic model that establishes a correlation between metabolite expression patterns and sample classifications, thereby facilitating accurate and refined forecasts of sample categorization. Unlike PCA, OPLS-DA serves as a supervised statistical approach for evaluating classification. This method ensures the neutralization of the influence of the independent variable X in the modeling process, thus accentuating variations in variable Y that are unrelated to data adjustments. Consequently, it enhances the emphasis placed on vital factors for classification while efficiently filtering out insignificant noise and removing superfluous variables, leading to a deeper comprehension of the model architecture and amplifying its analytical potency and reliability. An invaluable application of OPLS-DA lies in its capability to distinguish biochemical differences among diverse groups by pinpointing a VIP value associated with each metabolite. The higher the VIP score, the more significant role played by the compound in demarcating these contrasting groups. In the OPLS-DA analysis, we treated the substances as dependent variables and different samples as independent variables, which revealed that the model prediction index (Q^2) was 0.865, the fitting index of the independent variable (R^2_x) was 0.805, and the fitting index of the dependent variable (R^2_y) was 0.946 (Fig. 2A). Both R^2 and Q^2 were greater than 0.5, demonstrating that the model-fitting outcome is acceptable. After conducting 200 permutation tests, the intersection points between the Q^2 regression line and the vertical axis fell below 0 (Fig. 2B), indicating that the model did not exhibit signs of overfitting and appeared to be valid. Thus, the OPLS-DA analysis successfully discriminated MH-*Bc* from the other honey samples based on metabolic characterization.

The S-plot obtained from OPLS-DA analysis was utilized to facilitate the selection of marker compounds with the strongest and most reliable correlation (Fig. 2C). The abscissa of the S-plot displayed the covariance between the principal components and metabolites, while the ordinate represented the correlation coefficient. Generally, S-plots can assist in identifying compounds with significant differences by locating those closer to the upper right and lower left corners, ensuring a more objective and reliable assessment. After conducting a comprehensive analysis integrating statistical techniques and mass spectrometry identification, we found that TDBF with the accurate precursor ion 275.091 4 $[M+H]^+$ and product ions 260.051 2, 243.065 0, and 200.539 1 was detected in MH-*Bc* and plant-*Bc* samples but not in other types of honey. Moreover, as previous literature reported, TDBF was also detected in the methanolic extract of the *Bc* plant[36]. Thus, we considered TDBF as a potential marker for discriminating MH-*Bc*.

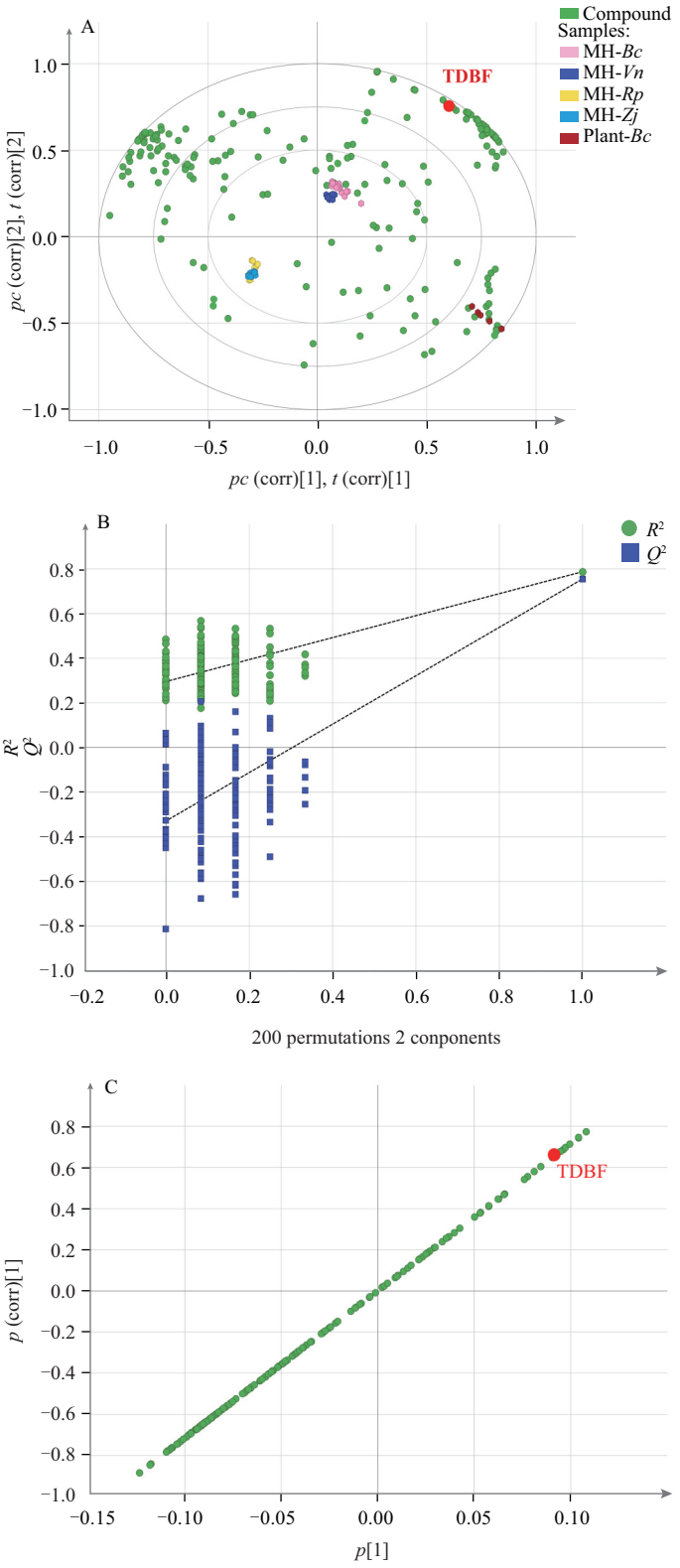

Fig. 2 UPLC-Q-TOF-MS/MS-based untargeted analysis for chemical marker identification. (A) Correlated analysis by OPLS-DA model among the monofloral honey samples of MH-*Bc*, MH-*Vn*, MH-*Rp*, and MH-*Zj*, as well as the plant samples of Plant-*Bc*. (B) Corresponding validation plot of the OPLS-DA model. (C) S-plot analysis of the OPLS-DA model. The statistical analysis was conducted using the SIMCA 14.1 software.

3.3 UPLC-QqQ-MS/MS-based targeted quantitative method validation and chemical marker quantification

To quantify TDBF in MH-*Bc*, we developed a novel UPLC-QqQ-MS/MS method where parameters including fragment voltage, collision energy, and quantitative and qualitative ion pairs were optimized for detecting TDBF (Table 1). Preliminary experiment results suggested that TDBF had a higher response under acid flow and when the MS was operating in the positive mode. Therefore, we selected 0.1% formic acid-acidified deionized water and 0.1% formic acid acidified acetonitrile as mobile phases in subsequent experiments and analyses.

In the positive ion mode, the precursor ion of TDBF was *m/z* 275.0 with product ions of *m/z* 260.0, *m/z* 243.0, and *m/z* 200.0. Among these, the *m/z* 243.0 ion was chosen as the quantitative ion due to its highest corresponding value (Fig. 3). The quantification of TDBF content in honey samples was performed using the external standard method[37]. We next evaluated the matrix effect by comparing the slopes of the matrix-matched and solvent-based calibration curves. The matrix effect was determined to be 17.10%. We adopted matrix-matched calibration curves to ensure quantitative accuracy. The matrix-matched calibration curves exhibited a linear range 2–300 μg/L with an R^2 value exceeding 0.99. The LOD was determined to be 0.5 μg/kg, while the limit of quantification (LOQ) was 2 μg/kg (Table 1).

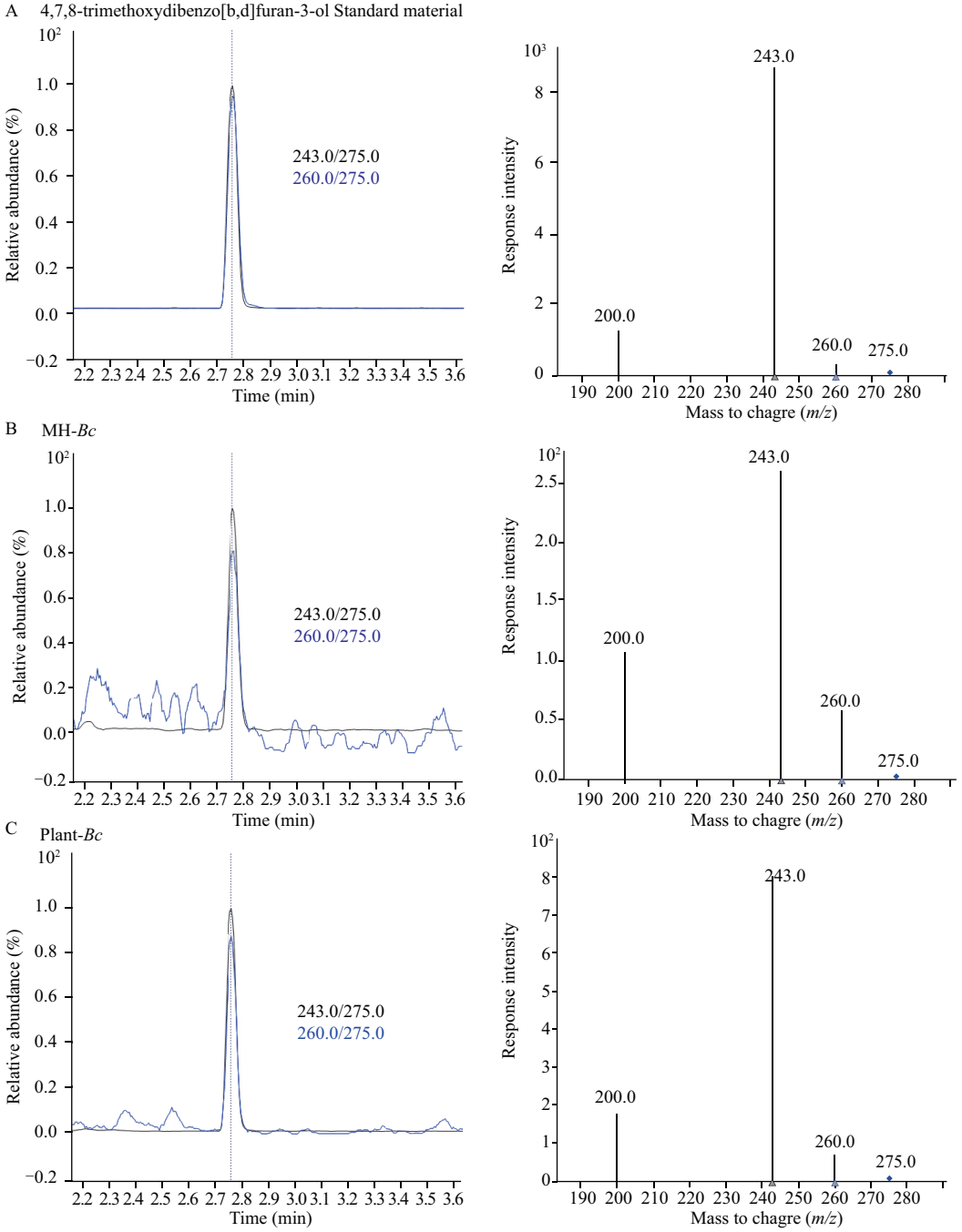

Fig. 3 UPLC-QqQ-MS/MS-based targeted analysis results of TDBF in samples. (A) MRM spectrum of TDBF standard. (B) MRM spectrum of TDBF in the monofloral honey sample of MH-*Bc*. (C) MRM spectrum of TDBF in the plant sample of Plant-*Bc*.

Table 3
The recovery and matrix effect for the quantification of TDBF by UPLC-QqQ-MS/MS, and the contents detected in real samples.

Recovery (%)						Matrix effect (%)	Content (μg/kg)					
2 μg/kg		4 μg/kg		40 μg/kg			MH-*Bc*	Plant-*Bc*	MH-*Am*	MH-*Zj*	MH-*Vn*	MH-*Rp*
Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day							
85.39 ± 1.47	83.80 ± 2.24	105.69 ± 2.68	111.07 ± 2.72	110.65 ± 1.96	108.56 ± 2.89	17.10	19–27	254–327	ND	ND	ND	ND

Note: ND means not detected. Mean ± RSD, n = 5.

To validate the accuracy of the established quantification method, the TDBF standard was added to blank honey samples at 3 different concentrations (2, 4, and 40 μg/kg). The intra-day precision was calculated based on the recovery of spiked samples analyzed three times on the same day, verifying the within-run replicates to reflect the repeatability of the assay. The inter-day precision reflects the repeatability of the method under different environmental and instrument conditions. The recovery rates of spiked samples were analyzed over three separate days, with measurements taken every 5 days. The intra-day precision was 85.39%–110.65%, with an RSD of 1.47%–2.68%. The inter-day precision values of TDBF ranged from 83.80% to 111.07% and the RSD ranged from 2.24% to 2.89% (Table 3). The above results conform to the standard specifications (RSD ≤ 3%)[38]. The previously reported findings have consistently validated the precision of our established quantitative approach.

3.4 Real sample analysis

According to the optimized and validated quantitative methods mentioned above, TDBF was quantified in 30 MH-*Bc* samples, 10 MH-*Vn* samples, 10 MH-*Rp* samples, 10 MH-*Zj* samples and 10 MH-*Am* samples, as well as 5 plant-*Bc* samples. The results indicated that concentrations of TDBF ranged from 19 to 27 μg/kg in MH-*Bc* samples while it was absent in other honey types (MH-*Vn*, MH-*Rp*, MH-*Zj* and MH-*Am*) (Tables 3 and S4). Therefore, TDBF can be considered a reliable chemical marker for distinguishing MH-*Bc* from different types of honey with confidence.

4. Conclusion

We combined untargeted and targeted mass spectrometry methods to identify and quantify a distinctive marker of MH-*Bc*. Through our analysis, we discovered that TDBF is a dependable chemical marker for identifying MH-*Bc*, a compound unique to MH-*Bc*. TDBF was not detected in other kinds of honey, making it an accurate and authentic characteristic marker for MH-*Bc*. The detection of TDBF can therefore efficiently ensure the quality and identity of genuine MH-*Bc*, thus holding immense importance for the future industrial production of MH-*Bc* product quality assurance.

Conflict of interest

The authors declared no potential con fl icts of interest with respect to the research, authorship, and/or publication of this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250346.

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