Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Insights from untargeted metabolomics: revealing potential marker compounds in the maturation of fermented chili

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A R T I C L E I N F O

Article history:
Received 20 June 2024
Received in revised form 26 July 2024
Accepted 17 December 2024

Keywords:
Fermented chili
Mature standards
Volatile substances
Non-volatile compounds
Multivariate statistical analysis

A B S T R A C T

Chili fermentation is one of the most important processes in the production of Pixian Douban (PXDB), which determines the flavor and product quality of PXDB. However, the maturity of fermented chili mainly depends on empirical judgment, which can not satisfy the need for standardized production of PXDB. Therefore, this study aimed to investigate volatile and non-volatile substances during the maturation process and to find markers related to the maturity of fermented chili. Two-dimensional gas chromatography-mass spectrometry (GC × GC-MS) combined with multivariate statistical analysis and relative odor activity values (ROAV) analysis revealed that 2-methoxy-3-isobutyl pyrazine, linalool, 3-(methylthio)propionaldehyde, myrcene, and decanal (ROAV ≥ 1, VIP > 1, $P < 0.05$) were regarded as potentially active aromatic markers for differentiating fermentation time. Additionally, ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) combined with multivariate statistical analysis revealed that 25 compounds could serve as differentiated non-volatile compounds. The correlation of maturity-related physicochemical indicators with volatile and non-volatile compounds revealed that four volatile compounds (2-methoxy-3-isobutylpyrazine, linalool, myrcene, and decanal) along with seven non-volatile compounds could serve as markers for evaluating the maturity of fermented chili. This study is expected to establish a standard for the determination of the maturity the fermented chili and lay the foundation for intelligent production of PXDB.

1. Introduction

The production of Pixian Douban (PXDB) is a globally acclaimed Chinese condiment, renowned for its unique flavor characteristics[1]. It plays a vital role in Sichuan cuisine, and its production techniques have been recognized as a batch of national intangible cultural heritage[2-3]. Generally, the production process of PXDB typically involves 3 fundamental steps. In the first stage, broad beans are mixed with wheat flour (17%, *m*/*m*) inoculated with *Aspergillus oryzae* to produce koji for 5–7 days. The koji of the broad bean is moved to a tank with salt and water and fermented for 2–3 months. In the second step, the cut chili (*Capsicum annuum* L.) is added with 15% salt and left at room temperature for 3–6 months[2]. Eventually, the meju and the salted chili are mixed at a ratio of 1:3 (*m*/*m*) and naturally ripened for at least 6 months to produce a distinctive flavor[4].

Chili fermentation is one of the most important processes in the production of PXDB, which determines the flavor and product quality of PXDB. However, the maturity of chili mainly relies on empirical judgment without reference to relevant standards, which can not satisfy the need for standardized production of PXDB. Therefore, it is important to look for key compounds related to maturation. The flavor of the chili

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Peer review under responsibility of Beijing Academy of Food Sciences.

SciOpen Publishing services by Tsinghua University Press

http://doi.org/10.26599/FSHW.2024.9250456

fermentation process is affected by the environment, microorganisms, and processing technology, and its flavor characteristics depend on volatile and non-volatile compounds, which are important sensory attributes of fermented chili[5-6]. Therefore, the analysis and characterization of these compounds can provide crucial insights for the quality assessment of fermented chili. Nevertheless, most studies have focused on the correlation between microbial community succession and key odor compounds during fermentation. Dong et al.[7] discovered that the predominant bacteria and fungi in the zha-chili fermentation process were *Lactobacillus* and *Kazachstania*, respectively. Furthermore, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeasts played a significant role in the development of flavor during chili fermentation. Peng et al.[8] revealed that microbial succession influenced the formation of volatile metabolites, with Lactobacillaceae showing a positive correlation to these metabolites during chili fermentation. Furthermore, several studies have investigated the effect of exogenous microorganisms (*Pichia* spp., *Pediococcus ethanolidurans* M1117) on the physicochemical properties and flavor formation of fermented chili[9-10]. Additionally, Liao et al.[11] observed that the salt content altered the microbial succession in fermented chili and affected the distribution of flavor compounds. However, few studies have focused on markers of mature chili and especially discussed volatile and non-volatile compounds during chili fermentation.

With the rapid development of detection technologies, untargeted metabolomics has emerged as an appealing approach for detecting and analyzing unknown metabolites in fermented foods[12-13]. This method allows for improved identification of unknown metabolites or metabolic patterns without the necessity of predefined scientific targets[14]. Metabolomics is increasingly recognized as an effective tool for characterizing the metabolism of fermented foods, such as fermented tea, dairy products[15] and grain[16], while also monitoring their dynamic changes[17]. Furthermore, advancements in metabolomics have facilitated the integration of chemometrics into the detection of key metabolites in fermentation, enabling the identification and quantification of specific biomarkers within the system under evaluation[12]. Therefore, it can be used to monitor the metabolite variation during fermentation and to find the markers of maturation in chili.

In this study, two-dimensional gas chromatography-mass spectrometry (GC × GC-MS) and ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) techniques were used to investigate volatile and non-volatile substances during fermentation. Multivariate statistical analysis was applied to try to find chemical markers related to the maturity of fermented chili of PXDB. This study provides a basis for better improvement of the current empirical-based judgment and lays a foundation for the standardization of chili fermentation.

2. Materials and methods

2.1 Materials and reagents

The chili (*Capsicum annuum* L.) was purchased from Wal-Mart supermarket (Sichuan, China). The salt was purchased from 9D Salt Co., Ltd. (Sichuan, China). 1,2-Dichlorobenzene, *n*-alkanes (C_8–C_{25}), methanol, and acetonitrile were obtained from Sigma-Aldrich Co., Ltd. (Shanghai, China). Chromatography grades formic acid and isopropyl alcohol were taken from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). All other reagents were of analytical grade.

2.2 Sample collection

The chili were destemmed and washed in a drum washer for 20 min. Subsequently, they were mashed and combined with 15% salt by weight and then fermented naturally in ceramic jars. The sample was regularly turned over every 3 days, ensuring that the chili inside was evenly stirred from top to bottom. Samples were collected randomly at 0, 14, 28, 42, 56, and 70 days, respectively. About 30 g of sample was collected from each of the three sampling points at the bottom, middle, and top of the vat, and then mixed evenly. The chili samples were stored at −80 °C for further study.

2.3 Total titratable acidity and determination of reducing sugar

Total titratable acidity was measured using 0.1 mol/L NaOH, yielding a titration endpoint at pH 8.2. Reducing sugar was determined by the National standard of China GB/T 5009.7—2016 *Determination of reducing sugars in food*. Specifically, the content of reducing sugar was measured by the method of direct titration.

2.4 GC × GC-MS analysis of volatile metabolites in chili samples

The volatile metabolites of chili samples were analyzed by using a GC × GC-MS 2010 NX system (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a DB-Wax column (30 m × 0.25 mm × 0.25 μm, Supelco, Inc., Bellefonte, PA, USA). Volatile substances were determined by the method of Xu et al.[18] with minor modifications. In brief, 5.00 g sample was placed in a pre-prepared headspace vial with 10 μL of 1,2-dichlorobenzene (1 μg/mL) as an internal standard, then the vial was sealed and equilibrated in a water bath at 60 °C for 20 min. The volatiles were then absorbed with solid-phase microextraction (SPME) fibers (CAR/PDMS, 75 μm) for 30 min. Subsequently, desorption of volatiles were achieved in the GC injector at 240 °C for 3 min. The analytical conditions were as follows: the temperature of the column was maintained at 40 °C for 2 min, ramped to 240 °C (held for 4 min) at 6 °C/min. The injection temperature and ion source temperature were 240 and 250 °C, respectively, and the scan range of MS was *m*/*z* 41–330.

2.5 Identification of volatile compounds

Identification of volatile compounds was based on comparing their retention indexes (RIs) and mass spectra with the standard database (NIST20 Library). The RIs were calculated by using a C_8–C_{25} *n*-alkane series. The odor threshold of each odorant was determined with reference to the compilation of odor[19]. The volatile components were semi-quantitative by the standard internal method to obtain each substance's relative abundance. All analyses were done in duplicate.

2.6 Relative odor activity values (ROAV) analysis

The contribution of each volatile compound to flavor depends on its particular odor threshold and concentration. Compounds with ROAV ≥ 1 are widely recognized as aroma-active compounds that can appreciably influence the overall aroma profile of a sample[20]. The ROAV calculation equation was listed as:

$$\mathrm{ROAV}_i = 100 \times \frac{C_i}{C_{\max}} \times \frac{\mathrm{OT}_{\max}}{\mathrm{OT}_i}$$

Where, C_i (ng/kg) was the relative content of volatile compounds; OT_i (mg/kg) was the aroma threshold in water for volatile compounds; $C_{\max}$ (ng/kg) is the highest relative concentration of the compound in the sample; and $\mathrm{OT}_{\max}$ (mg/kg) is the largest odor threshold of the compound.

2.7 *UPLC-QTOF-MS analysis of non-volatile compounds in chili samples*

2.7.1 *Metabolite extraction*

The metabolites extraction and UPLC-QTOF-MS analysis were performed according to Espichán et al.[21]. The chili sample (100 mg) was extracted by ultrasonic with 800 μL methanol solution (80%) and subjected to vortexing for 1 min and then extracted with ultrasound for 15 min at 25 °C. Subsequently, the samples were centrifuged at 14 000 r/min for 10 min at 4 °C. Then 600 μL of the supernatant was transferred to a vial to obtain supernatants ready for injection.

2.7.2 *UPLC-QTOF-MS conditions*

Liquid chromatographic separations were performed using UPLC-20A (SHIAMDZU, Japan) coupled to an X500R mass spectrometry system (SCIEX, USA). The compounds were separated by an Acquity UPLC BEH C_{18} (2.1 mm × 100 mm, 1.7 μm, Waters, USA). The column temperature was 30 °C, the injection volume was fixed at 3 μL with a flow rate of 0.3 mL/min. Mobile phase A was formic acid aqueous solution (0.1%, *V/V*), and mobile phase B was acetonitrile solution (0.1%, *V/V*). The gradient elution was as follows: 95% A, 5% B for 5 min; 5% A, 95% B for 10 min.

MS conditions were as follows: ion spray (IS) voltage 4 500 V; source temperature 550 °C; The ion source is heated electrospray (HESI) with ion source gas I (GSI), curtain gas (CUR), and gas II (GSII) at 55, 25, and 55 psi, respectively, and the scan range was *m/z* 100–1 000.

2.8 *Identification of non-volatile substances*

Raw data were converted using Reifycs Abf Converter V 4.0. After conversion, mass spectrometry analysis was performed using MS-DIAL V 5.0 for peak detection, deconvolution, and alignment. Identification was carried out also in MS-DIAL software by comparing the MS/MS information with the public MS/MS database in positive. The tolerances for MS and MS/MS comparing were set to 0.01 Da and 0.025 Da, respectively. Retention time and MS tolerance of peak alignment were set to 0.05 min and 0.015 Da, respectively.

2.9 *Statistical analysis*

All tests were repeated three times. Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) of SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Duncan's test was performed for multiple comparisons under the level of significance of $P < 0.05$. The statistical analysis results were plotted using Origin 8.0 software.

3. Result and discussion

3.1 *Analysis of maturity-related indicators*

Total titratable acidity content in fermented chili exhibited a gradual increase followed by stabilization after 56 days (Fig. 1a). Growth and metabolism of microorganisms produce organic acids, resulting in an increase in titratable acidity[22]. After 56 days of fermentation, the total titratable acid content in the fermentation system attained a dynamic equilibrium, indicating that the fermented chili had reached maturity. Simultaneously, the changes in reducing sugars were evaluated as they serve as a vital carbon source for microbial growth and metabolism, influencing the maturation process (Fig. 1b). The levels of reducing sugars displayed a gradual increase followed by a decline, reaching their lowest point at 70 days ((3.87 ± 0.10) g/100 g). The increase in reducing sugars was believed to be due to the LAB decomposing sucrose into reducing sugars, and then these sugars were subsequently utilized as carbon sources in fermented vegetables, ultimately leading to a decrease in reducing sugars[23]. This finding was consistent with the research conducted by Li et al.[24].

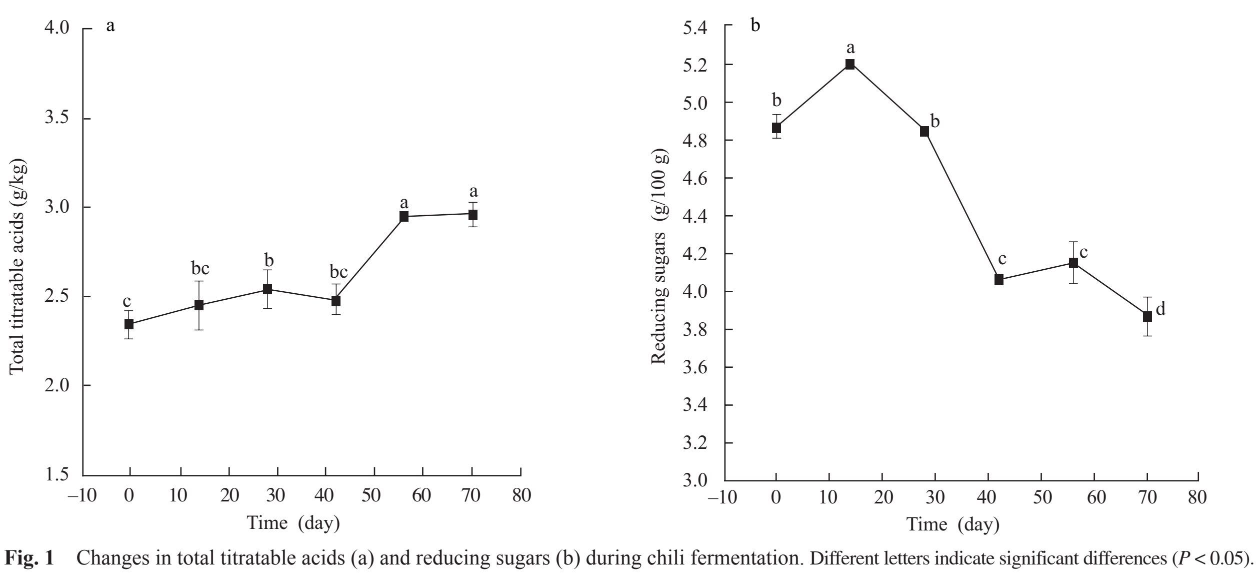

Fig. 1 Changes in total titratable acids (a) and reducing sugars (b) during chili fermentation. Different letters indicate significant differences ($P < 0.05$).

3.2 Exploring potential volatile markers of chili during fermentation

3.2.1 Analysis of volatile substances during the fermentation process

Generally, the content and composition of aroma compounds also varied with the ripening process. Therefore, GC × GC-MS was used to examine volatiles at different stages. Eighty-two compounds were detected during fermentation (Table S1), including esters, aldehydes, alcohols, ketones, alkenes, acids, phenols, and other compounds in chili samples. Alcohols, aldehydes, and esters dominated the eight compound categories (Fig. 2a), with alcohols being the most prevalent at 29.3%. Phenols and acids were a small group of components. Alcohols were a metabolite of microorganisms during the fermentation process, mainly derived from the oxidation of lipids and degradation of carbohydrates, imparting an aromatic quality to chili vegetables[25]. Esters, which are the result of the reaction between alcohols and acids during fermentation, possess sweet or fruity flavor and enhance the flavor of fermented foods by lowering the intensity of unpleasant odors[26-27]. Aldehydes, with low odor thresholds contribute significantly to the overall flavor of the product[28].

The total content of volatile compounds in naturally fermented chili was relatively high at the beginning and end of fermentation, with a slight decrease observed in the middle stage of fermentation (Fig. 2b). Olefins showed an increasing trend in the chili fermentation stage. It was possible that the high levels of olefins in the raw material of chili peppers, coupled with the metabolic effects of microorganisms, led to a gradual predominance. Despite the high olefin content in volatile components, its contribution to the aroma was limited due to high thresholds[29]. This was consistent with Li et al.[24].

As the fermentation proceeded, the alcohol content showed a decrease, but it still remained at approximately 18% at the end. Alcohols, including 1-hexanol, 3-hexen-1-ol, 2-heptanol, and alpha-terpineol, play a significant role in both fresh and fermented chili by contributing to the sweet, refreshing, and pungent flavors[30]. In addition to contributing flavor, alcohols are also precursors for the production of esters and other aroma components. The production of esters is assumed to be a signal of maturation in fermented food[31]. It was worth noting that esters were predominantly synthesized in the early stages of fermentation, leading to higher levels at that time. The high concentration of esters, such as methyl salicylate, methyl hexadecanoate, methyl linoleate, and methyl linolenate, contributes to the sweet and fruity flavor of savory chili and is likely formed by the metabolism of amino acids or the oxidative metabolism of fatty acids[32]. Methyl salicylate possessed a mint flavor and was distinguished by the highest concentration of esters, corroborating Xiao et al.[33].

The aldehyde concentration decreased during fermentation, reaching a minimum of 64.29 ng/g at 42 days, approximately 34.8% of the initial concentration. Aldehydes, particularly those with low odor thresholds, have been found to play a crucial role in influencing the unique flavor profile of the product[28], as they are often linked to the oxidative degradation of unsaturated fatty acids and the Strecker degradation of amino acids[34].

To determine whether fermentation processes can be classified by volatile organic compounds, the heatmap analysis was visualized based on the abundance of the volatile metabolites. As illustrated in Fig. 3, samples from days 14 and 28 were closely clustered together, indicating a higher similarity in their volatile compounds. On the other hand, the chili samples at day 0 were clustered separately. These results illustrated that the times of fermentation had a greater impact on the volatile components of chili.

3.2.2 Analysis of potential volatile markers during the process of fermented chili

Orthogonal partial least squares-discriminant analysis (OPLS-DA) is an efficient way to explore the specific characteristics of volatile compounds profiles[35]. Based on the OPLS-DA model, the aroma changes of chili during fermentation could be obviously divided (Fig. 4a). The model parameters (R^2_Y = 0.981, Q^2 = 0.929) indicated a good fit with high predictiveness of differential metabolites across comparative groups. To prevent overfitting of the OPLS-DA model, the model was validated using the replacement test, and the results showed that no overfitting occurred in the model built from the data of each stage (Fig. 4b). The initial fermented chili samples were in quadrant 3, samples on day 70 were in quadrant 4, and the others were in quadrants 1 and 2. The results suggested that the maturation of fermented chili may be evaluated using the OPLS-DA model.

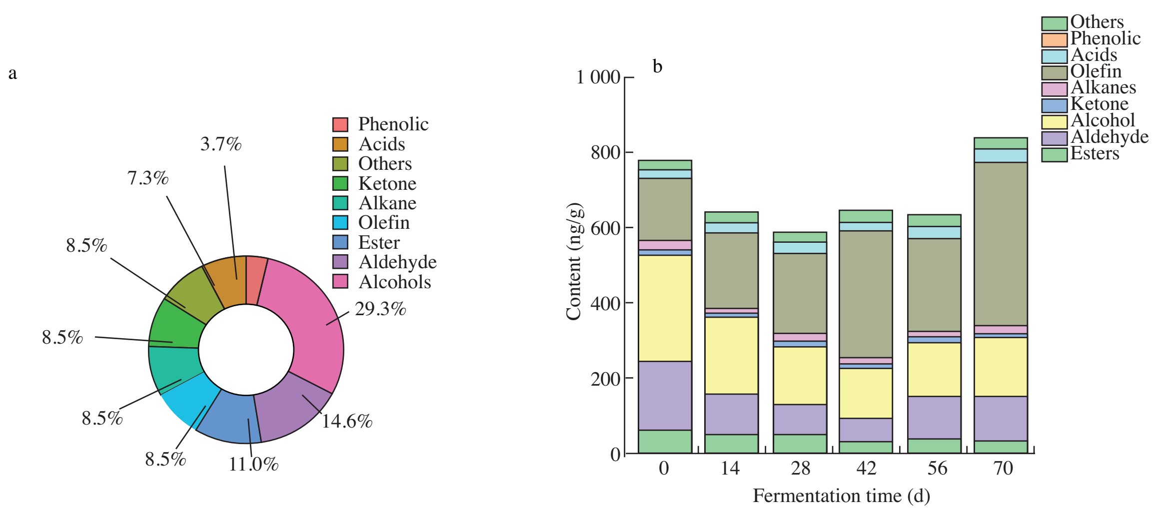

Fig. 2 The proportion of volatile compounds (a) and content of volatile compounds in different stages of fermented chili (b).

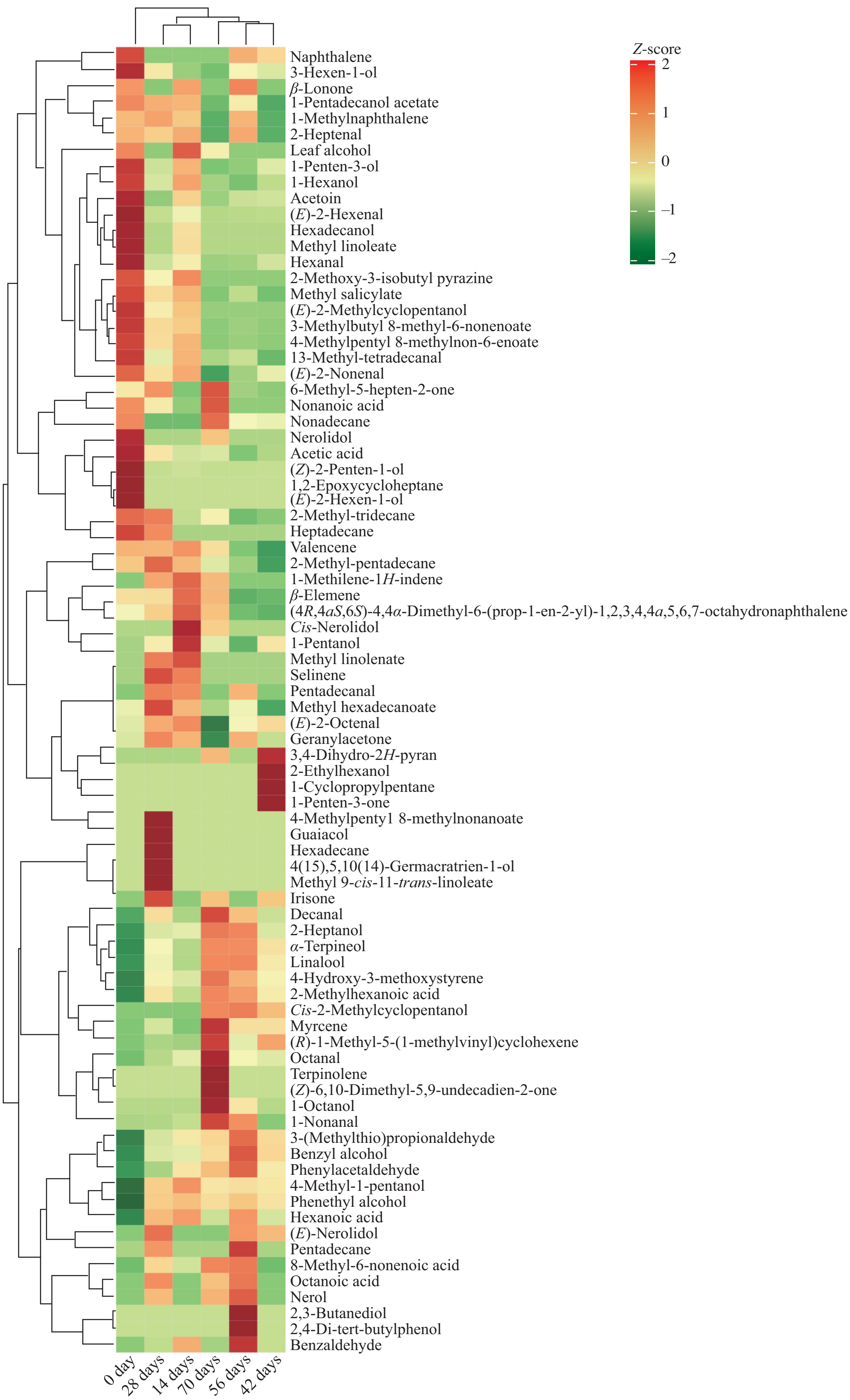

Fig. 3 Heatmap of volatile compounds in fermented chili at different stages (the variation of colors in each square indicates the changes in relative concentrations of volatile compounds at the different fermentation stages. Red squares indicate a higher content of compounds, while green squares indicate a lower content).

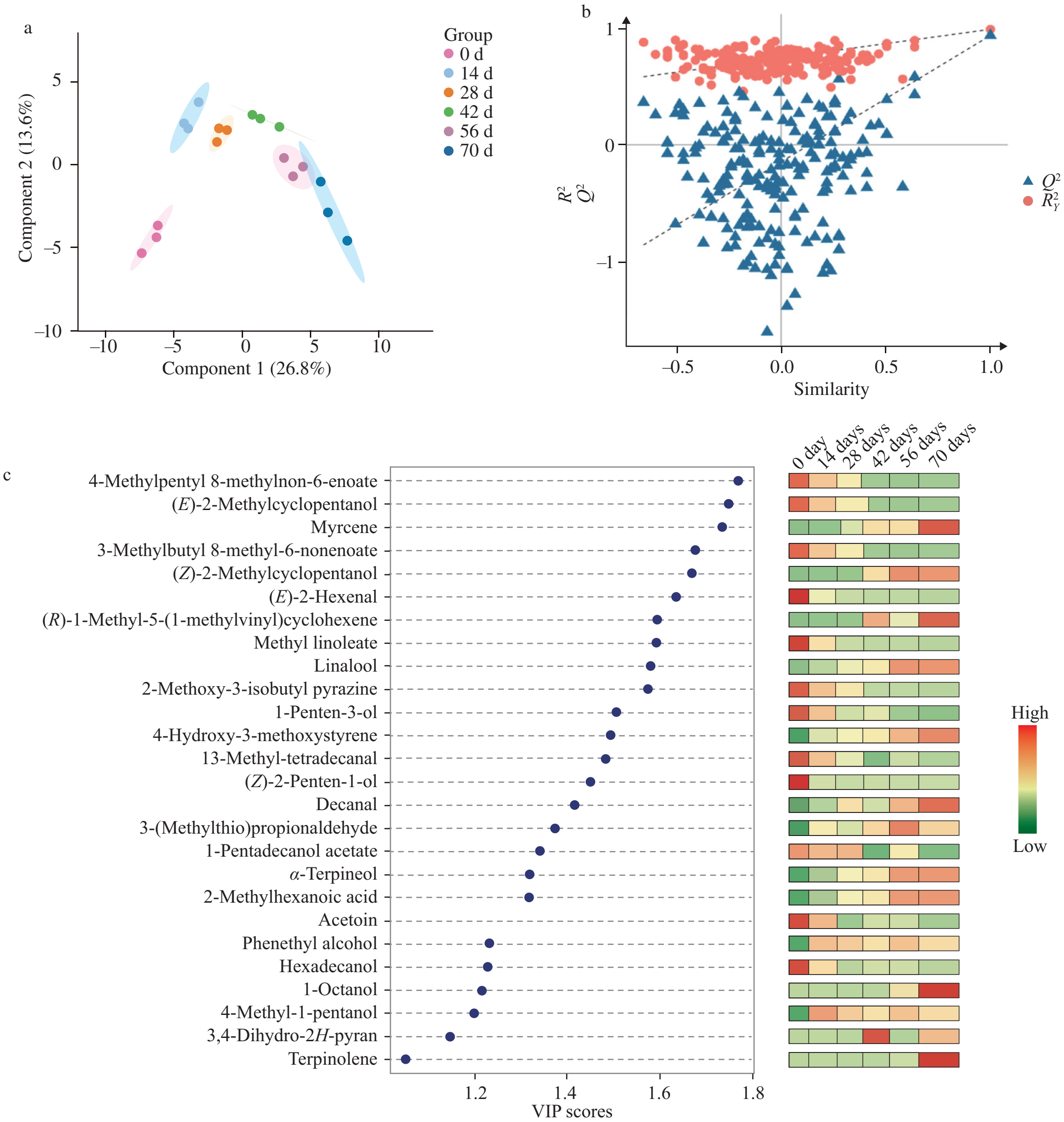

Fig. 4 OPLS-DA of volatile compounds in fermented chili at different stages (a); Model validation permutation test for OPLS-DA of volatile compounds (b). Heat map of differential volatile compounds (c).

A significant difference test and variable importance in the projection (VIP) of volatile compounds contents among fermented samples were carried out by multivariate statistical methods. In this study, 26 volatile compounds were successfully identified using a VIP score > 1.0 and P < 0.05 as screening conditions, including 10 alcohols, 4 esters, 3 aldehydes, 3 olefins, an acid, a phenol, a ketone. VIP denotes the variable projection importance, with higher values indicating greater significance and a more substantial influence on predictive performance. Notably, 4-methylpentyl 8-methylnon-6-enoate (VIP = 1.77), (*E*)-2-methyl cyclopentanol (VIP = 1.75) and myrcene (VIP = 1.73) exhibited the highest VIP scores, suggesting that they exhibited more significant differences across various fermentation stages.

Fig. 4c indicated that 14 main compounds, such as myrcene, decanal, linalool, 3-methylthiopropionaldehyde, α-terpineol, and 1-octanol, increased across fermentation stages, most of which contribute sweet and floral flavors to the products. Conversely, the levels of 12 compounds, including (*E*)-2-hexenal, 2-methoxy-3-isobutylpyrazine, 1-penten-3-ol, (*Z*)-2-penten-1-ol, methyl linoleate, and hexadecanol, which primarily contributed to the green flavor of fermented chili, showed a decline. These compositional shifts in volatile compounds suggest a substantial influence on the overall flavor profile of the fermented chili.

To further identify aroma activity markers during the maturation process, ROAV is utilized to assess their impact on the overall aroma of chili. It is typically defined that compounds with ROAV ≥ 1 contribute to the overall aroma of the product, playing a pivotal role in the fermented chili's overall aroma[36]. As shown in Table 1, five volatile flavor compounds with ROAV ≥ 1, including myrcene, 2-methoxy-3-isobutyl pyrazine, linalool, decanal, and 3-(methylthio) propionaldehyde.

Specifically, 2-methoxy-3-isobutyl pyrazine exhibited high ROAV in 0–28 days of chili fermentation. It is noteworthy that 2-methoxy-3-isobutyl pyrazine, despite its relatively low concentration, showed a green flavor profile with a low threshold value (0.000 006 2 mg/kg), and contributed significantly to the predominant flavor. In addition, pyrazines have been extensively studied in the context of grapes and

Table 1
ROAV of differential volatile flavor compounds.

CAS	Compounds	Odor description	OT (mg/kg)	0 day	14 days	28 days	42 days	56 days	70 days
6728-26-3	(*E*)-2-Hexenal	Apple, green	0.087 7	0.37	0.11	0.14	0.26	0.19	0.17
1576-95-0	(*Z*)-2-Penten-1-ol	Green, plastic, rubber	0.72	0.01	< 0.01		< 0.01		
111-87-5	1-Octanol	Chemical, metal, burnt	0.11					0.02	0.07
616-25-1	1-Penten-3-ol	Butter, pungent, grassy	0.358 1	0.01	0.01	0.01	0.02	0.01	0.01
24683-00-9	2-Methoxy-3-isobutyl pyrazine	Earth, spice, green pepper	0.000 006 2	100.00	56.56	100.00			
4536-23-6	2-Methylhexanoic acid		0.92	< 0.01	< 0.01	0.01	0.02	0.02	0.02
3268-49-3	3-(Methylthio)propionaldehyde	Cooked potato	0.000 45	2.41	8.09	16.62	46.08	48.05	37.02
7786-61-0	4-Hydroxy-3-methoxystyrene	Clove, curry	0.012 02		0.03	0.08	0.18	0.19	0.23
626-89-1	4-Methyl-1-pentanol		0.82		0.01	0.02	0.03	0.03	0.03
513-86-0	Acetoin	Butter, cream	0.014	0.10	0.08	0.15	0.39	0.30	0.28
112-31-2	Decanal	Soap, orange peel, tallow	0.003	0.29	0.46	1.52	2.72	2.95	3.75
78-70-6	Linalool	Flower, lavender	0.000 22	8.67	14.76	42.13	100.00	100.00	100.00
123-35-3	Myrcene	Balsamic, must, spice	0.001 2			1.40	5.54	4.39	9.83
586-62-9	Terpinolene	Pine, plastic	0.2						0.01
98-55-5	*α*-Terpineol	Oil, anise, mint	1.2	< 0.01	< 0.01	< 0.01	0.01	0.01	0.01
6728-26-3	(*E*)-2-Hexenal	Apple, green	0.087 7	0.37	0.11	0.14	0.26	0.19	0.17

wine, with research indicating that they are a significant contributor to the green flavor profile in wines[37]. As fermentation proceeds, linalool, 3-(methylthio)propionaldehyde, myrcene and decanal were the key flavor compounds, providing floral, fruity aromas and cooked potato flavors to fermented chili. Among these, linalool exhibited the highest ROAV after 28 days of fermentation, adding floral and lavender aroma to fermented products. It has been identified as an aromatic compound in various fermented products[38]. Extracellular enzymes produced by fungal growth during fermentation have been identified as playing a crucial role in the synthesis of monoterpene alcohols in the fermentation process[39]. Furthermore, linalool served as a crucial biosynthetic precursor for other floral aroma compounds. Myrcene contributes an herbaceous and pungent flavor and plays a key role in the aroma profile of fermented chili[40]. The rise in terpene aglycones like linalool and myrcene might be linked to the hydrolysis of terpene glycosides during fermentation[33].

3-(Methylthio)propionaldehyde was the second-highest in activity at 42–70 days of fermentation, imparting a potato-like aroma. 3-(Methylthio)propionaldehyde, which may originate from the breakdown of methionine, is a byproduct of the Strecker degradation of methionine during fermentation processes. The fermentation process caused various volatile compounds to alter, resulting in the fermented chili changing from a strong green aroma to a distinctive strong floral, fruity, and potato aroma, which also marked the maturity of the chili. Therefore, it is considered that the above five compounds, including myrcene, 2-methoxy-3-isobutyl pyrazine, linalool, decanal, 3-(methylthio)propionaldehyde (ROAV ≥ 1, VIP > 1, $P < 0.05$) played an essential role in the ripening process of fermented chili.

3.3 Exploring potential non-volatile markers of chili during fermentation

3.3.1 Analysis of non-volatile compounds of the fermentation process

The non-targeted UPLC-QTOF-MS technique was employed to evaluate the changes in non-volatile components of chili during fermentation, as detailed in Table S2. A total of 7 647 metabolite ion signatures were detected in electrospray ionization (ESI) positive ion mode, and 79 compounds in chili samples were identified through a qualitative analysis based on MS^2 spectroscopy and metabolomics databases, including amino acids and their derivatives, cinnamic acids, flavonoids, vitamins, coumarins, organic acids, reducing sugars, nucleosides, and alkaloid compounds.

Among these, 8 amino acids and their derivatives were detected, including arginine, isoleucine, phenylalanine, tryptophan, tyrosine, valine, and cyclo(proline-leucine) and 4-acetamidobutanoate. Fresh chili typically contains about 10% protein by dry weight and is a major source of amino acids[41]. Amino acids are not only the basic units of proteins but also precursors of taste and aroma[42]. Furthermore, six cinnamic acid compounds and their derivatives, such as ferulate, *trans*-ferulic acid, moupinamide, coumaric acid, methyl *trans*-cinnamic acid, and (*E*)-*N*-caffeoyl putrescine, have been identified in this study, which aligns with previous findings establishing ferulic acid as one of the major polyphenols in fresh chili[43]. A total of eight flavonoids were observed, which included chalcones, flavones, and isoflavones. Moreover, seven alkaloids were identified, specifically harmane, capsaicin, nordihydrocapsaicin, dihydrocapsaicin, tabersonine, piperlotine A, and brucine. Capsaicin is known to be the main cause of a tingling sensation in the mouth. Capsaicin and dihydrocapsaicin are the primary constituents of CAPs, accounting for nearly 90% of the overall CAP content[41]. These compounds account for a high proportion of all non-volatile compounds and are the four major categories of non-volatile compounds.

3.3.2 Screening of potential non-volatile markers in the maturation process

To understand the differences among compounds and between each sample group, the data were analyzed with multivariate statistical methods (Fig. 5a). The OPLS-DA model parameters exceeded 0.9 in all cases, suggesting that the model is capable of explaining and predicting variations among non-volatile compounds at various fermentation stages. To further assess whether any overfitting occurred in the model, a cyclic replacement test with 200 iterations was conducted, and the outcomes are illustrated in Fig. 5b. The intersection points of the Q^2 regression line with the Y-axis fell

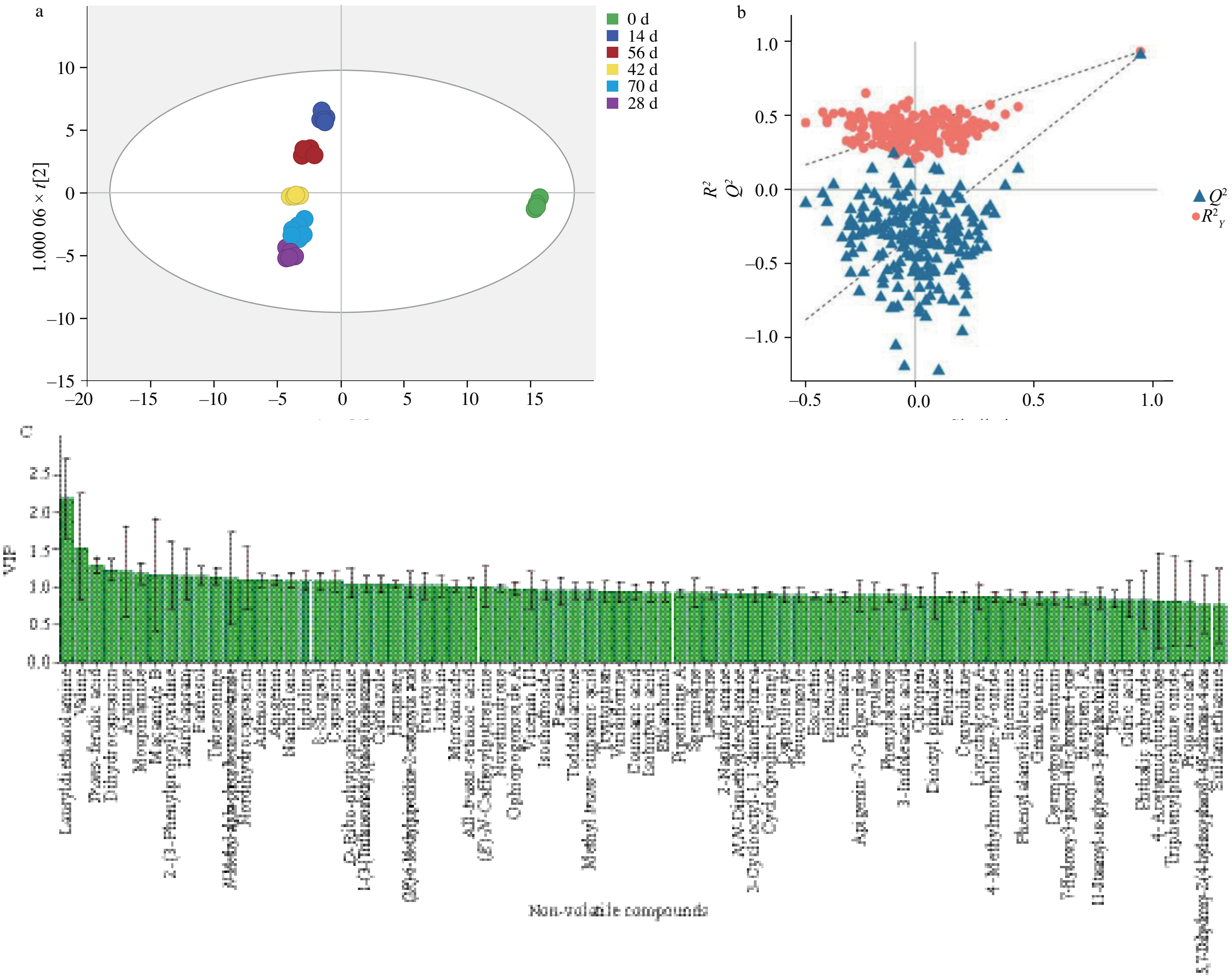

Fig. 5 OPLS-DA of non-volatile compounds in fermented chili at different stages (a) and model validation permutation test for OPLS-DA (b), VIP values of non-volatile compounds (c).

entirely within the negative half of the axis, suggesting that the OPLS-DA model is both stable and reliable, with no evidence of overfitting. Employing selection criteria of VIP > 1.0 and $P < 0.05$, 25 differential non-volatile compounds were identified across various fermentation stages. Among these, lauryldiethanolamine (VIP = 2.19), valine (VIP = 1.53), and *trans*-ferulic acid (VIP = 1.30) demonstrated the highest VIP values, as visualized in Fig. 5c, while the relative abundance profiles of all differential non-volatile compounds throughout the fermentation progression are comprehensively presented in Fig. 6.

Five differential alkaloids were found in the OPLS-DA model. It is especially noteworthy that capsaicin and dihydrocapsaicin, known as the main compounds responsible for the intense heat sensation in the mouth, experienced a reduction in concentration as ripening progressed. Similarly, fermentation is acknowledged for its capability to reduce the spiciness of chili[44]. Microorganisms present in fermented chili degraded capsaicin by producing capsaicin acylase, which leads to the formation of vanillin amine and 8-methoxyvinic acid as carbon sources[45]. Separately, capsaicin is a base that can be neutralized in a highly acidic fermentation environment. Consequently, fermented chili generally displays a milder, more appealing aromatic pungency compared to fresh chili[46]. During the fermentation process, the levels of trans-ferulic acid exhibited an initial increase followed by a decline, showing variations over time. This observation is consistent with the findings of Vong et al.[47], who reported a reduction in ferulic acid content in chili peppers following lactic acid bacteria fermentation, leading to the formation of novel phenolic derivatives. Free amino acids exhibit both biological functionality and distinct taste characteristics. Specifically, the content of arginine, which imparts a bitter taste, decreased throughout fermentation, whereas valine demonstrated an initial increase followed by a subsequent decline. Apigenin and luteolin were considered as differentially expressed flavonoid compounds possessing various biological activities[48], and their concentrations were observed to increase during the fermentation process, consistent with the findings of earlier study[49]. Soluble sugars primarily are in the forms of sucrose, glucose, and fructose during chili, and fructose is the most abundant. Throughout the fermentation process, a notable reduction in fructose levels was observed, attributable to its role as a primary carbohydrate converted into lactic acid, which subsequently contributes to the development of the flavor and aroma in the fermented vegetables[23]. Adenosine is an endogenous nucleoside, a compound in which the N-9 position of adenine and the C-1 position of *D*-ribose are connected through a glycosidic bond. Aaslyng et al.[50] believed that the breakdown and release of nucleotides gives the meat an additional flavor.

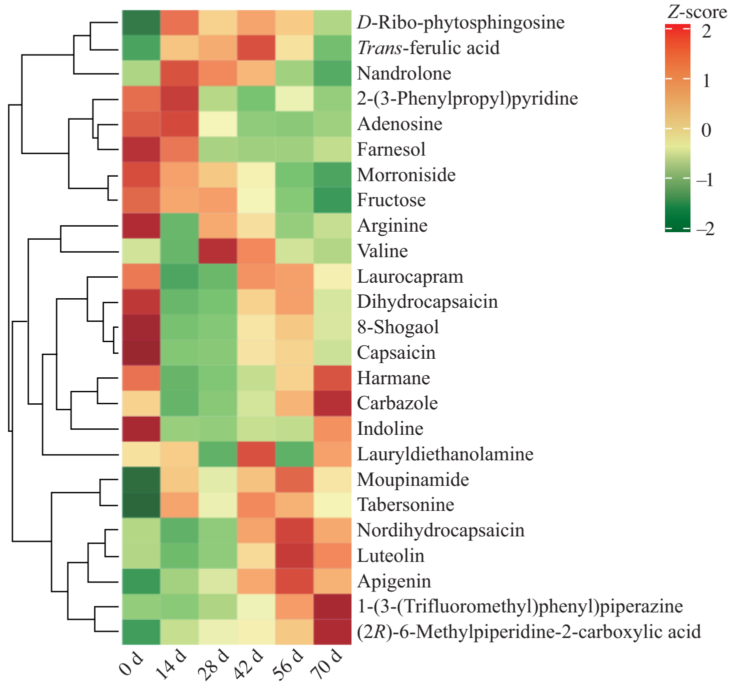

Fig. 6 Heat map of differential non-volatile compounds. The red color indicates a high abundance of pairs and the green color indicates low relative abundance.

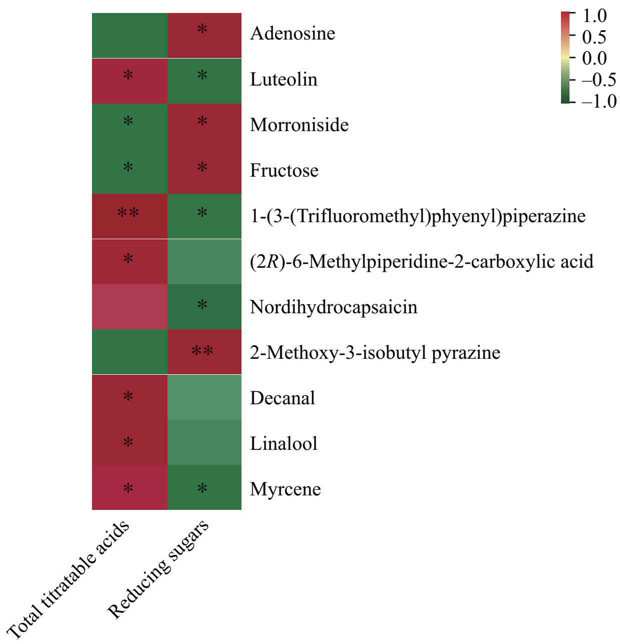

Fig. 7 Correlation analysis of metabolites with physical and chemical indicators. *$P < 0.05$; **$P < 0.01$.

3.4 *Identification of maturation marker compounds*

Correlation analyses were used to examine potential associations between the screened differential compounds and maturity attributes to identify the compounds with the greatest degree of association. As illustrated in Fig. 7, among the 5 differential aromatic volatiles, 4 compounds demonstrated a significant correlation with total titratable acids or reducing sugar. Specifically, 2-methoxy-3-isobutylpyrazine exhibited a strong positive correlation with reducing sugars and a moderate negative correlation with total acids. Both linalool and decanal exhibited a strong positive correlation with total titratable acid. It is noteworthy that myrcene exhibited a strong and significant positive correlation with total titratable acids, while demonstrating a strong and significant negative correlation with reducing sugars. Additionally, differential non-volatile compounds demonstrated varying degrees of correlation with total titratable acids or reducing sugars. There was a significant positive correlation between adenosine and reducing sugar, while (2*R*)-6-methylpiperidine-2-carboxylic acid exhibited a strong positive correlation with total acidity. Remarkably, luteolin, 1-(3-(trifluoromethyl)phenyl)piperazine, fructose, and morroniside demonstrated significant correlations with both total acids and reducing sugars. Therefore, we hypothesized that four volatile compounds (2-methoxy-3-

isobutylpyrazine, linalool, decanal, and myrcene) and seven non-volatile compounds (adenosine, (2*R*)-6-methylpiperidine-2-carboxylic acid, nordihydrocapsaicin, luteolin, 1-(3-(trifluoromethyl) phenyl)piperazine, fructose, and morroniside) could serve as markers for evaluating the maturity of chili.

4. Conclusions

This study investigated both volatile and non-volatile compounds in fermented chili by GC × GC-MS and UPLC-QTOF-MS. Twenty-six volatile differential compounds were identified based on VIP > 1 and $P < 0.05$, with 5 compounds regarded as potentially active aromatic markers for differentiating fermentation time. Capsaicin, dihydrocapsaicin, *trans*-ferulic acid, adenosine, apigenin, fructose, etc., could be considered as potential non-volatile markers in the fermentation process. The correlation analysis revealed that 4 volatile compounds (2-methoxy-3-isobutylpyrazine, myrcene, linalool, decanal) and seven non-volatile compounds, including adenosine, (2*R*)-6-methylpiperidine-2-carboxylic acid luteolin, fructose, and morroniside could serve as markers for evaluating the maturity of fermented chili. This study is expected to establish a standard for the determination of the maturity of fermented chili and lay the foundation for the intelligent production of PXDB. Future research should focus on in-depth determination of volatile and non-volatile substances in different batches of fermented chili, and modelling of the data from a large number of samples in order to validate key markers that are closely related to chilli maturity.

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.

Acknowledgments

This work was supported by the Sichuan Provincial Department of Science and Technology (2024ZHCG0086) and the Chongqing Science and Technology Commission (cstc2021jscx-cylhX0014).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250456.

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