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Dong, Ling

2022-12-30

Dong , L , Zhao , C , Zhang , F , Ma , Y , Song , C , Penttinen , P , Zhang , S & Li , Z 2022 , ' Metabolic characterization of different-aged Monascus vinegars via HS-SPME-GC-MS and CIL LC-MS approach ' , Lebensmittel - Wissenschaft und Technologie , vol. 172 . https://doi.org/10.1016/j.lwt.2022.1

http://hdl.handle.net/10138/352808
https://doi.org/10.1016/j.lwt.2022.114169

LWT - Food Science and Technology 172 (2022) 114169

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Metabolic characterization of different-aged *Monascus* vinegars *via* HS-SPME-GC-MS and CIL LC-MS approach

Ling Dong [a,g,h,1], Chi Zhao [a,1], Fengju Zhang [a], YingLun Ma [b], Chuan Song [c,d], Petri Penttinen [e,f], Suyi Zhang [c,d,**], Zhihua Li [a,c,d,*]

[a] Institute of Agro-products Processing Science and Technology, Sichuan Academy of Agricultural Sciences, Chengdu, Sichuan, 610066, China
[b] Fujian Yongchun Old Vinegar Industry Co., Ltd., Quanzhou, 362000, China
[c] Luzhou Laojiao Co., Ltd., Luzhou, 646000, China
[d] National Engineering Research Center of Solid-state Brewing, Luzhou, 646000, China
[e] College of Resources, Sichuan Agricultural University, Chengdu, 611130, China
[f] Faculty of Agriculture and Forestry, University of Helsinki, 00014, Finland
[g] Horticulture Institute, Sichuan Academy of Agricultural Sciences, Chengdu, 610066, Sichuan, China
[h] Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Southwest Region), Ministry of Agriculture, Chengdu, 610066, Sichuan, China

ARTICLE INFO

Keywords:
Aging process
Antioxidant capacities
Volatile components
Non-volatile metabolites
Principal component analysis

ABSTRACT

Yongchun *Monascus* vinegar is one of famous Chinese vinegar types because of its unique flavor and special bioactivity. Aging process has been regarded as crucial for enhancing the flavor and quality of vinegar. However, changes in the metabolites along the aging of the vinegar are still poorly understood. In this study, a combination of headspace solid-phase micro-extraction gas chromatography-mass spectrometry and chemical isotope labeling liquid chromatography-mass spectrometry methods was used for investigating the metabolomes of one-year-old, five-year-old and thirty-year-old YMVs. DPPH radical-scavenging activity, total phenolics content, and total flavonoids content correlated positively with the aging time. The metabolite compositions in the different-aged vinegars were clearly separated in the PCA analysis. A total of 1133 volatile and non-volatile metabolites changed along the aging; 392 metabolites were in common whereas 126, 84, and 54 changed metabolites were unique to one-to-five year, one-to-thirty year, and five-to-thirty year-old vinegar comparisons, respectively. Organic acids and dipeptides, exhibiting taste characteristics, with constant increase or decrease with aging time and correlated with antioxidant capacities could be used as biomarkers for differentiating the different-aged vinegars. The results revealed aging time related changes in volatile and non-volatile metabolites in YMVs, providing useful knowledge for improving their quality.

1. Introduction

Vinegars have attracted much attention due to their unique taste and beneficial effects on health, e.g., antioxidant, anti-inflammatory, anti-fungal and antibacterial activities, and antidiabetic effects and cardio-vascular diseases effects (Chen, Chen, Giudici, & Chen, 2016; Kandylis, Bekatorou, Dimitrellou, Plioni, & Giannopoulou, 2021). Yongchun *Monascus* vinegar (YMV), one of the four famous traditional Chinese vinegar types, has a unique flavor, special bioactivity and more than 2000 years of history (Yuan, Chen, Virk, Ma, & Chen, 2021; Zhang, Huang, Zhou, & Wu, 2017). Different from the other traditional Chinese cereal vinegars, YMV is produced using liquid fermentation, the main raw material is glutinous rice, and the starter for saccharification and alcohol fermentation is Hongqu, i.e., a *Monascus* spp. culture on glutinous rice (Ai et al., 2019). After liquid fermentation, YMV is stored in a jar or pot (Fig. 1) for a long time to enhance the flavor, taste, and health beneficial characteristics according to a national standard in China (Product of Geographical Indication – Yongchun aged vinegar, GB/T 26531–2011) (Fang, Chai, Zhong, & Jiang, 2021).

Recently, the analysis of flavor, metabolites and microbial communities in cereal vinegar products has received considerable attention (Ai

* Corresponding author. Institute of Agro-products Processing Science and Technology, Sichuan Academy of Agricultural Sciences, Chengdu, Sichuan, 610066, China.
** Corresponding author. National Engineering Research Center of Solid-state Brewing, Luzhou, 646000, China.
E-mail addresses: zhangsy@lzlj.com (S. Zhang), lzh82@hotmail.com (Z. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.lwt.2022.114169
Received 21 June 2022; Received in revised form 8 November 2022; Accepted 14 November 2022
Available online 16 November 2022

Abbreviations

HS-SPME-GC-MS	headspace solid-phase micro-extraction gas chromatography-mass spectrometry
CIL LC-MS	chemical isotope labeling liquid chromatography-mass spectrometry; DPPH, 2,2-diphenyl-1-picrylhydrazyl
TPC	total phenolics content
TFC	total flavonoids content
YMVs	Yongchun *Monascus* vinagars
PCA	principal component analysis
GR	glutinous rice
FAAs	free amino acids
FRP	fermented red pepper
LC-UV	liquid chromatography coupled to ultraviolet
CAR/PDMS	carboxy/polydimethylsiloxane
RIs	retention indices
UPLC	ultra performance liquid chromatography
PLS-DA	partial least squares discriminant analysis
VIP	variable importance for the projections
CBS	cystathionine β-synthase
SCFAs	short-chain fatty acids
DAT	desaminotyrosine
NOAEL	no-observed-adverse-effect level

Fig. 1. Yongchun *Monascus* vinegar fermentation vessels at Fujian Yongchun Old Vinegar Industry Co., Ltd. Samples (n = 3) were collected from each batch.

et al., 2019; Al-Dalali et al., 2020; Jiang et al., 2019). For example, in vinegar acetic acid played an essential role and contributed to the sour taste and pungent smell. Various free amino acids (FAAs) and organic acids, e.g., lactic acid, propanoic acid, butanoic acid, citric acid, and glycolic acid have been considered to improve the taste of vinegar (Fang et al., 2021; Kandylis et al., 2021; Zhao et al., 2020). In our previous study, 1285 metabolites were identified in five cereal vinegars (Li, Dong, et al., 2022). However, metabolic changes in aging the vinegar, especially *Monascus* vinegar, in long-term storage are still poorly understood.

Chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS) analysis gives in-depth information with high-accuracy on submetabolomes based on chemical groups, e.g., chemical derivatization different chemical groups (Li, Dong, et al., 2022; Zhao, Li, Han, Chan, & Li, 2019). CIL LC-MS method has been successfully utilized to analyze the metabolites in various food, such as cereal vinegar (Li, Zhao, et al., 2022), fermented red pepper, soy sauce and tofu (Li, Dong, et al., 2022), and milk (Mung & Li, 2018).

In this study, a combination of the head-space–solid-phase micro-extraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS) and CIL LC-MS methods was used to investigate differences in volatile and non-volatile metabolites among *Monascus* vinegars of different ages. The total concentrations of metabolites were quantified by liquid chromatography coupled to ultraviolet (LC-UV) spectroscopy, and high amounts of concentration metabolites present in *Monascus* vinegars were putatively identified. Furthermore, the metabolic pathway of metabolites with significantly changed and their correlation with antioxidant capacities were revealed. The results reveal the changes in metabolites along the aging of Yongchun *Monascus* vinegar and provide useful knowledge for improving the quality of *Monascus* vinegars.

2. Materials and methods

2.1. Chemicals and reagents

Gallic acid (purity >98%) and rutin (purity >97%) were purchased from Shanghai Yuanye Biotechnology Co Ltd. (Shanghai, China). Analytical grade Folin-Ciocalteu reagent, Na_2CO_3, $AlCl_3$ and CH_3COOK were purchased from Chengdu Kelong Chemistry Reagent Co Ltd. (Chengdu, China). LC-MS grade water, methanol (MeOH), acetonitrile (ACN), 0.1% formic acid (FA) in water, 0.1% FA in ACN, *n*-alkanes (C8–C20) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, purity >97%) were purchased from Sigma-Aldrich Shanghai Trading Co Ltd. (Shanghai, China) or Sigma-Aldrich Canada Co. (Markham, ON, Canada). 4-channel labeling kits (NMT-4101-KT for amine/phenol, NMT-4123-KT for acid, NMT-4145-KT for hydroxyl, and NMT-4167-KT for carbonyl metabolomics) were purchased from Nova Medical Testing Inc. (Edmonton, AB, Canada, www.novamt.com).

2.2. Sample collection

The different aged sample were selected based on different criteria which are price in market and antioxidant activity. Three batches of Yongchun *Monascus* vinegar samples, aged for 1, 5, and 30 years at 20–28 °C, were obtained from Fujian Yongchun Old Vinegar Industry Co., Ltd. (Quanzhou, China). The total acidity and pH were measured according to the National Food Safety Standard in China (GB 5009.235–2016). Briefly, 2 mL vinegar was mixed with 18 mL deionized water, followed by measuring the pH with a FE28 pH meter (Mettler Toledo, Shanghai, China) and titrating with 0.1 mol/L NaOH solution till pH 8.2. The total acidity was expressed as the acetic acid content (g/100 mL). Unless otherwise mentioned, all analyses were performed in triplicate.

2.3. DPPH radical-scavenging activity, TPC and TFC

To measure 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, 5 mL vinegar was mixed with 95 mL 80% (v/v) MeOH. To measure total phenolics content (TPC) and total flavonoids content (TFC), 5 mL vinegar was mixed with 95 mL 70% (v/v) MeOH. DPPH radical-scavenging activity, TPC and TFC were determined at 517 nm, 765 nm and 415 nm, respectively, using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) as described previously (Gao et al., 2021) and expressed as the percentage radical-scavenging activity (% RSA), mg gallic acid equivalents (GAE) L^{-1} and mg rutin

Table 1
Antioxidant activities of one, five and thirty year-old Yongchun *Monascus* vinegars.

YMV age (years)	DPPH scavenging activity (%)	Total phenolics content (mg GAE mL^{-1})	Total flavonoids content (mg RE mL^{-1})
1	7.12 ± 0.34^{Bc}	0.76 ± 0.002^{Bc}	0.18 ± 0.002^{c}
5	12.12 ± 0.026^{Bb}	0.94 ± 0.006^{Bb}	0.23 ± 0.001^{b}
30	44.76 ± 0.221^{Aa}	1.47 ± 0.016^{Aa}	0.32 ± 0.008^{a}

Different upper- and lower-case superscript letters in a column indicate statistically significant differences at $p < 0.01$ and $p < 0.05$, respectively.

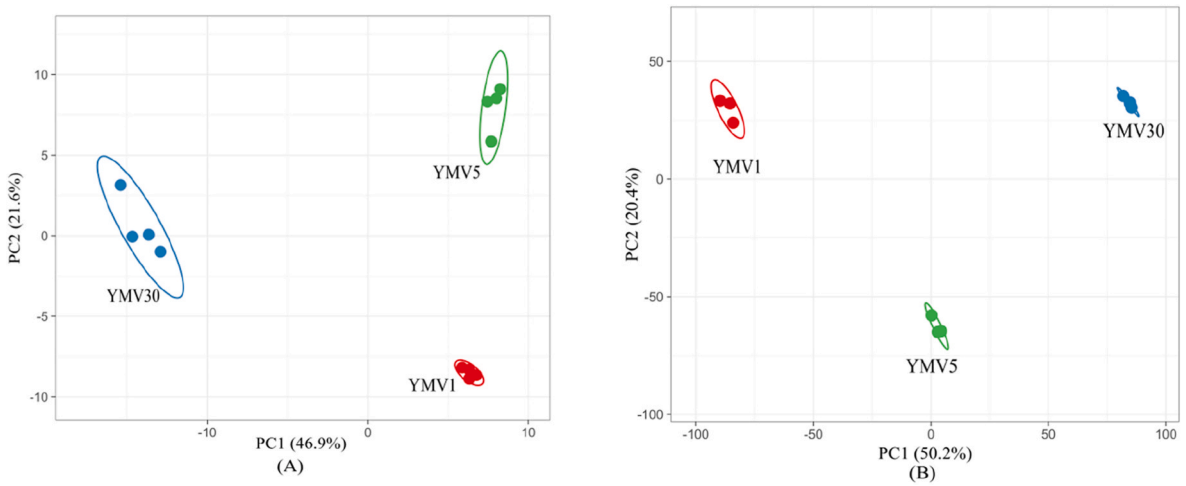

Fig. 2. Principal component analysis (PCA) of metabolites in one-year-old (YMV1), five-year-old (YMV5) and thirty-year-old (YMV30) Yongchun *Monascus* vinegar. A) Metabolites detected using HS-SPME-GC-MS; B) A total of 10,580 peak pairs from CIL-LC-MS.

equivalents (RE) L^{-1}, respectively.

2.4. HS-SPME-GC-MS analysis

A 75 μm carboxy/polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, PA, USA) was used to extract volatile compounds with full release (1 cm) from the head-space of a 20 mL vial containing 8 mL vinegar mixed with 2 g NaCl, pre-equilibrated at 45 °C for 30 min, as described previously (Al-Dalali et al., 2020) with some modifications. After extraction, a combiPAL RSI 85 autosampler (CTC Analytics, Zwingen, Switzerland) was used to insert the fiber into the injection port of a gas chromatography–mass spectrometry (GC-MS) system at 230 °C for 5 min of desorption. GC-MS was performed using an Agilent GC 9000 coupled to 5977 B mass selective detector (MSD) (Agilent Technologies, St Louis, MA, USA). The separation was carried out on a 30 m × 0.25 mm × 0.25 μm DB-WAX column (Agilent Technologies, USA). The carrier gas (helium) flow rate was 1.0 mL min^{-1}. The column temperature was initially maintained at 40 °C for 3.5 min, raised from 40 °C to 90 °C at 5 °C min^{-1}, held at 90 °C for 10 min, then raised from 90 °C to 230 °C at 12 °C min^{-1}, and maintained at 230 °C for 10 min. The MSD conditions were as follows: injector temperature was 250 °C, electron energy 70 eV, and mass scan range 33–450 amu. GC-MS raw data was analyzed using MS-DIAL with default parameters according to the manual (Tsugawa et al., 2015). Volatile compounds were identified by comparing their MS data and retention indices (RIs) determined using a series of *n*-alkanes (C8–C20).

2.5. CIL LC-MS analysis

The total concentration of labeled samples was measured using an Agilent 1290 ultra performance liquid chromatography (UPLC) system with a photodiode-array detector (PDA) as described previously (Mung & Li, 2018).

CIL LC-MS analysis was performed as described previously (Li, Dong, et al., 2022; Zhao et al., 2019). Briefly, a sample was diluted to 2 mmol/L and then split into four 15 μL aliquots to analyze amine/phenol, acid, hydroxyl, and carbonyl group based submetabolomes with ^{12}C-reagents isotope labeling. In addition, a pooled sample with 50 μL from each vinegar was mixed and an equal volume of ^{12}C-/^{13}C- reagent-labeled mixture was used as an internal standard for absolute quantification because of light (^{12}C) and heavy (^{13}C) labeled derivatives having nearly identical physical and chemical properties, including retention time. All samples were labeled using 4-channel labeling kits according to the SOP of the kits. Metabolites were separated and detected using an Agilent eclipse plus reversed-phase C18 column (150 × 2.1 mm, 1.8 μm particle size) and Agilent 1290 LC linked to Bruker Impact II QTOF Mass Spectrometer, respectively. The mobile phase included solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The LC gradient was 25% B from 0 to 10 min, 99% B from 10 to 13.1 min, 25% B from 13.1 to 16 min, and it ended at 25% B. The flow rate was 400 μL min^{-1}. The column oven temperature was 40 °C and a mass range 220–1000 *m/z* was scanned. The raw mass data was exported to. csv file with Bruker Data Analysis 4.4. Data analysis including peak pair picking, alignment, zero-filling, filtering, and imputation was performed using IsoMS Pro 1.2.5 (NovaMT Inc.). Peak pairs were retained for further analysis when the peak pair had been detected in at least 80% of the same-group samples. Metabolites were identified by searching against an in-house mass spectral library NovaMT Metabolite Database v2.0 based on a three-tier ID approach. For tier 1, there are more than 1500 authentic metabolite standards and identification is based on the accurate mass and retention time (RT) matches. For tier 2, there are over 9000 metabolites with predicted RT and metabolic pathway related information on each metabolite for high-confidence putative identification in NovaMT Metabolite Database v2.0 (Zhao et al., 2019).

2.6. Statistical analysis

Principal component analysis (PCA) was carried out by a web tool ClustVis (https://biit.cs.ut.ee/clustvis/) (Metsalu & Vilo, 2015). Partial least squares discriminant analysis (PLS-DA) was carried out by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) using the parameters "Auto scaling" and cross-validation (LOOCV) (Pang et al., 2021). One-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) was performed to test differences in physicochemical properties, organic acids and volatiles. Differences were considered statistically significant at $P < 0.05$. The relationships between antioxidant capacities and differential metabolites, DPPH radical-scavenging activity, TPC, TFC and metabolites with variable importance in the projection (VIP) > 1 in PLS-DA and $P < 0.05$ in a *t*-test were assessed in a correlation analysis in SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The volatile and non-volatile metabolites with statistically significant differences between the vinegars of different age were visualized in a Venn diagram. The metabolites VIP >1 in PLS-DA and $P < 0.05$ in a *t*-test were used as inputs for pathway analyses. The pathway analysis was performed using MetaboAnalyst with the Kyoto Encyclopedia of Genes and Genomes (KEGG) as the backend knowledgebase (Pang et al., 2021). The experiments were performed in triplicate and the results are expressed as mean value± standard deviations.

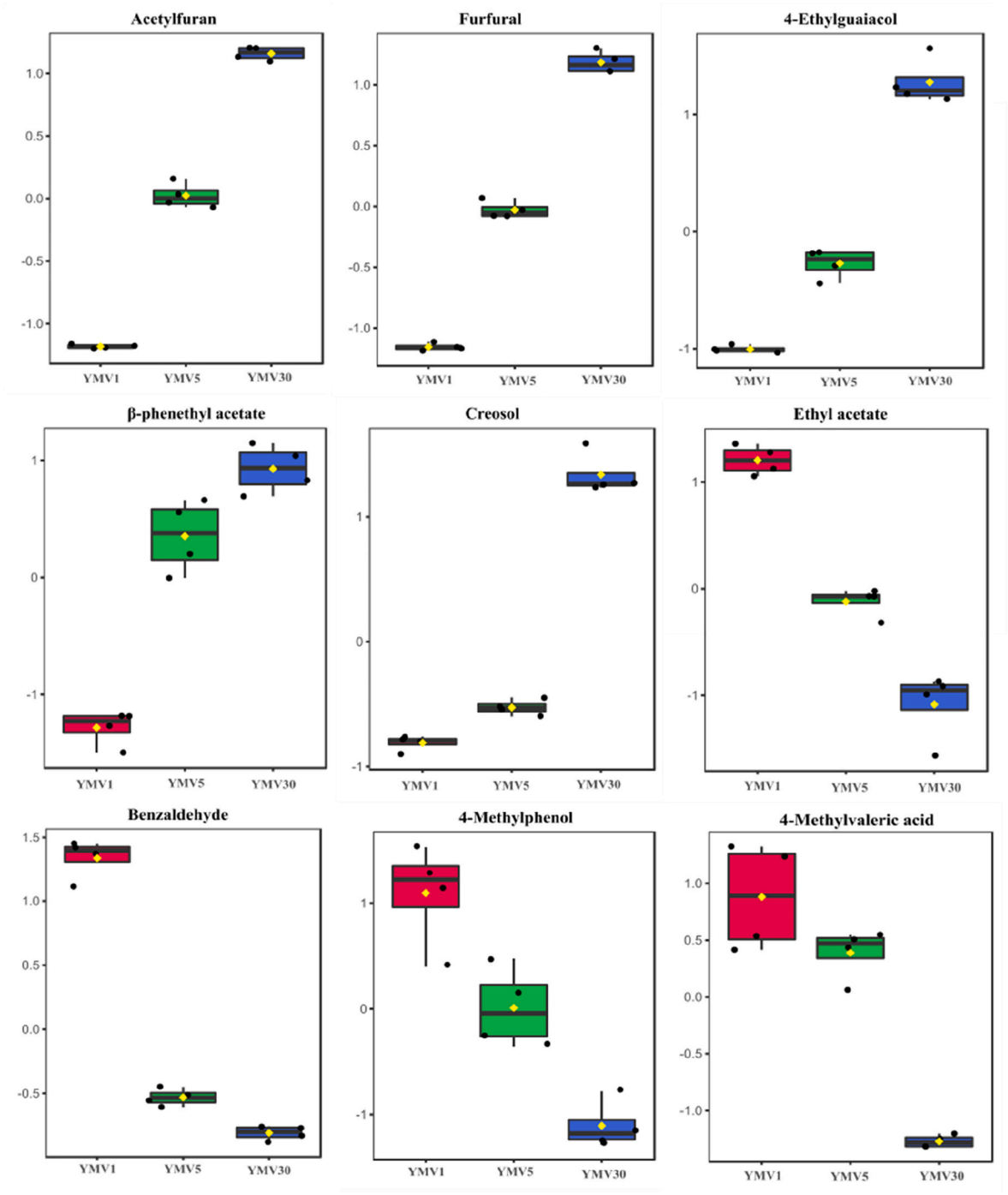

Fig. 3. Box and whisker plots of volatile metabolites with constant increase or decrease over the aging time in one-year-old (YMV1), five-year-old (YMV5) and thirty-year-old (YMV30) Yongchun *Monascus* vinegar. The exact values (mean ± SD) are in the Supplemental Table S1.

3. Results and discussion

3.1. Acidity, DPPH radical-scavenging activity, TPC, and TFC

Generally, vinegar with higher total acidity is regarded as having higher quality. The total acidity of *Monascus* vinegars should be equal to or higher than 5.0 g/100 mL (Chinese national standard GB/T 26531–2011). In this study, the total acidity of one-year-old ($n = 3$), five-year-old ($n = 3$) and thirty-year-old ($n = 3$) Yongchun *Monascus* vinegars were 6.10 ± 0.00 (pH = 3.96 ± 0.01), 6.02 ± 0.15 (pH = 3.90 ± 0.01), and 6.43 ± 0.03 (pH = 3.87 ± 0.01), respectively.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, total phenolics content (TPC) and total flavonoids content (TFC) of the vinegars were higher the older the Yongchun *Monascus* vinegar ($p < 0.05$) (Table 1). Notably, compared to the one-year-old vinegar, the DPPH value was over six times higher in the 30-year-old vinegar (Table 1). The results showed that similar to persimmon vinegar (Zou, Wu, Yu, Xiao, & Xu, 2017), Zhenjiang aromatic vinegar (Duan, Xia, Zhang, Li, & Zhang, 2019) and *Monascus* rice vinegar (Gao et al., 2021), the antioxidant activity in Yongchun *Monascus* vinegar increased with aging time.

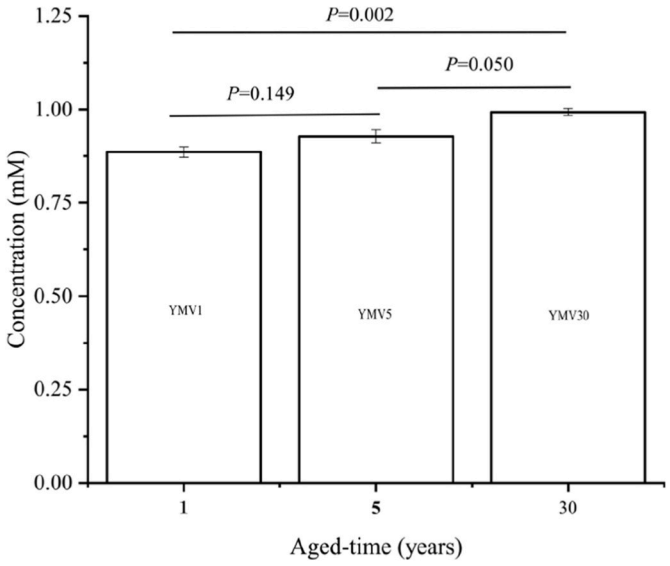

Fig. 4. LC-UV measurement of the total metabolite concentration in one-year-old (YMV1), five-year-old (YMV5) and thirty-year-old (YMV30) Yongchun *Monascus* vinegar.

3.2. HS-SPME-GC-MS analysis

The volatile compound compositions in the Yongchun *Monascus* vinegars were clearly different in the one-year, five-year and thirty-year-old vinegars (Fig. 2A). A total of 27 volatile compounds were identified, including benzene and substituted derivatives, organooxygen compounds, fatty acyls and phenols (Supplemental Table S1). The ion intensities of nine compounds changed over aging time ($p < 0.05$) (Fig. 3), which was probably connected to their participation in important physicochemical reactions, such as oxidation, esterification, hydrolysis, caramelization and Maillard reactions (Zhou et al., 2020).

The ion intensities of acetylfuran, furfural, 4-ethyl-guaiacol, β-phenethyl-acetate and creosol were the higher the older the vinegar (Fig. 3). Acetylfuran, contributing to sweet, almond, caramel, and balsamic taste, has been regarded as a characteristic metabolite that distinguishes aged vinegars (Nam, Lee, Kim, Song, & Jang, 2019), and identified in miso, sauce, and baijiu (He et al., 2020). Furfural, connected with caramel, almond, and burnt sugar flavor, is mainly generated during the decoction process with ribose, xylose, arabinose, galacturonic acid, glucuronic acid and pentosan as its and main precursors in the Maillard reaction (Gong et al., 2021). Furtherly, this volatile compound correlated positively with the relative abundances of *Acetobacter* and *Komagataeibacter* in the acetic acid fermentation stage of Shanxi aged vinegar production (Zhu et al., 2018). 4-Ethyl-guaiacol, contributing to sweet, spicy and smoky taste and belonging to aroma-active phenols, has been identified in fermented foods such as doubanjiang and soy sauce and may be utilized to improve the flavor quality (Feng et al., 2015). In our study, 4-ethyl-guaiacol increased with aging, whereas during the aging of Shanxi aged vinegar its content decreased (Liang et al., 2016); the difference may result from the different raw materials and fermentation conditions. β-Phenethyl-acetate is a fruity-tasting compound (Zhang, Wang, Xu, Wang, & Zhao, 2019) and correlated positively with *Komagataeibacter medellinensis* and *Yarrowia lipolytica* in *Monascus* vinegar (Jiang et al., 2019). In addition, creosol (4-methylguaiacol, spicy) and 4-ethylguaiacol not only contribute to the overall flavor but also exhibit antioxidant activities (Supplemental Table S4) and potent anti-inflammatory effects in baijiu (Hong, Zhao, & Sun, 2021).

The ion intensities of ethyl acetate that contributes to fruity flavor, benzaldehyde, 4-methyl-phenol, and 4-methyl-valeric acid correlated negatively with aging time (Fig. 3). Benzaldehyde, contributing to almond and cherry-like flavor, is easily converted to alcohols or acids, which may lead to a significant decline in its content during the aging process (Wang et al., Zhao et al., 2019). Overall, the results indicated that the flavor-contributing compounds changed significantly during the long-term aging process, thus affecting the quality of *Monascus* vinegar.

3.3. CIL LC-MS analysis

The metabolite concentration in Yongchun *Monascus* vinegars was lower in the one-year-old vinegar than in the thirty-year-old ($p < 0.05$) (Fig. 4). In total, 10,580 peak pairs were detected in the submetabolomes with an average of 9791 ± 199 (n = 9) peak pairs per sample. Similar with the volatile compound compositions, submetabolome compound compositions were clearly different in the one-year, five-year and thirty-year-old vinegars (Fig. 2B).

A total of 1353 metabolites, including 329 and 1024 metabolites in tier 1 and tier 2, respectively (Supplemental Table S2), were identified. The tier 1 metabolites were reduced by merging the isomers or different

Table 2
The highest relative ion intensity metabolites identified by CIL LC-MS.

Metabolite*	HMDB	Relative ion intensity (× 10^8)			Identification Level
		YMV1	YMV5	YMV30	
1,4-diaminobutane	HMDB01414	29.96 ± 2.49^a	30.70 ± 2.89^a	24.06 ± 2.24^b	Tier 1
Alanine	HMDB00161	17.84 ± 0.71^b	25.15 ± 1.35^a	23.92 ± 1.44^a	Tier 1
Cadaverine	HMDB02322	20.35 ± 1.60^{ab}	22.25 ± 2.54^a	17.48 ± 1.05^b	Tier 1
Proline	HMDB00162	10.59 ± 0.53^b	11.94 ± 0.68^a	12.54 ± 0.51^a	Tier 1
Leucine	HMDB00687	8.96 ± 0.49^a	9.81 ± 0.50^a	9.85 ± 0.57^a	Tier 1
Glycine	HMDB00123	7.87 ± 0.26^b	11.04 ± 0.97^a	11.58 ± 0.23^a	Tier 1
Tyramine	HMDB00306	13.16 ± 0.84^{ab}	13.75 ± 0.69^a	11.78 ± 0.52^b	Tier 1
Valine	HMDB00883	5.73 ± 0.22^b	8.22 ± 0.60^a	8.93 ± 0.19^a	Tier 1
γ-aminobutyric acid	HMDB00112	11.42 ± 0.58^a	9.34 ± 0.04^b	6.38 ± 0.16^c	Tier 1
Glutamic acid	HMDB00148	9.48 ± 0.51^a	2.51 ± 0.24^b	1.97 ± 0.11^b	Tier 1
Phenylalanine	HMDB00159	3.36 ± 0.10^a	3.27 ± 0.22^a	2.62 ± 0.19^b	Tier 1
Aspartic acid	HMDB00191	0.95 ± 0.59^c	1.91 ± 0.19^b	2.96 ± 0.17^a	Tier 1
Threonine	HMDB00167	2.51 ± 0.08^b	3.16 ± 0.39^a	2.79 ± 0.17^{ab}	Tier 1
Serine	HMDB00187	0.88 ± 0.06^b	0.98 ± 0.08^b	1.92 ± 0.11^a	Tier 1
4-Hydroxyphenylethanol	–	3.80 ± 0.11^{ab}	4.12 ± 0.38^a	3.59 ± 0.11^b	Tier 2
Choline	HMDB00097	0.41 ± 0.02^c	0.58 ± 0.12^b	0.93 ± 0.08^a	Tier 1
Lysine	HMDB00182	0.26 ± 0.01^c	0.41 ± 0.06^b	0.86 ± 0.07^a	Tier 1
Tyrosine	HMDB00158	0.45 ± 0.02^b	0.36 ± 0.03^b	0.87 ± 0.11^a	Tier 1
4-Aminobutyraldehyde	–	0.24 ± 0.01^c	0.27 ± 0.02^b	0.61 ± 0.01^a	Tier 2

"*" indicates peak pairs were detected from amine/phenol functional group. HMDB is the Human Metabolome Database. The different superscript letters within a row indicates a statistically significant difference at $p < 0.05$ (n = 3, mean ± SD).

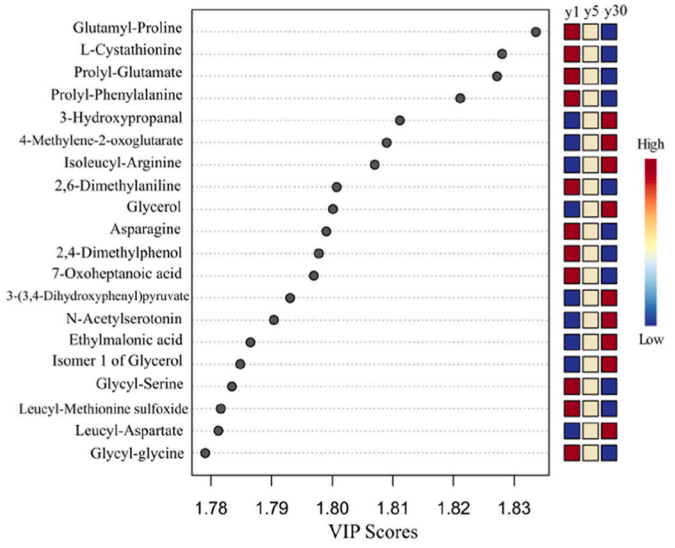

Fig. 5. The 20 metabolites with highest VIP scores in the partial least squares discriminant analysis (PLS-DA) of metabolites in one-year-old (y1), five-year-old (y5) and thirty-year-old (y30) Yongchun *Monascus* vinegar.

tags into 275 metabolites out of which 204 were organic acids and derivatives. Among them, 186 metabolites were in the subclass amino acids, peptides, and analogues (Supplemental Table S3). In CIL LC-MS, the MS peak intensity of a labeled metabolite could reflect the corresponding concentration because of the equalization of metabolite ionization efficiency (Zhao & Li, 2020). Accordingly, the labeled derivatives with high ion intensities were considered as metabolites with high abundances in the vinegars. The metabolites with highest relative ion intensities included 1,4-diaminobutane, alanine, cadaverine and proline (Table 2). In addition, other amino acids and organic acids were abundant in the vinegars. Cadaverine and 1,4-diaminobutane (putrescine) are polyamines that may be produced by the decarboxylation of amino acids and have been found in high concentrations in various fermented foods such as fermented vegetables, fish sauces, and cheese (del Rio et al., 2019). The level of cadaverine requires attention since high amounts of cadaverine are harmful for health; for rats, the oral toxicity of cadaverine was >2000 mg/kg body weight (del Rio et al., 2019).

3.4. Major changes in the metabolites

The PLS-DA model, based on the identified 1353 metabolites, showed goodness of prediction value Q2 = 0.955 and R2 = 0.999, indicating that PLS-DA model clearly separated the samples based on the age of the vinegars. Among the twenty metabolites with highest VIP-values, nine metabolites increased and eleven decreased with the aging time (Fig. 5). The twenty metabolites included thirteen amine/phenol, four hydroxyl, two carboxyl and one ketone/aldehyde group metabolites. The results indicated that esterification and/or oxidation as well as hydrolysis played important roles in the aging process. Similar with the aging process in Zhenjiang aromatic vinegar (Zhou et al., 2020), no clear change was observed in the total amount of aldehydes during aging. The amount of 3-hydroxypropanal (reuterin) increased during the aging process. Reuterin is a promising bio-preservative and therapeutic agent that may reduce colorectal cancer growth (Bell et al., 2022) and a broad-spectrum antimicrobial produced by lactobacilli such as *Lactobacillus reuteri* through glycerol dehydratase during fermentation. Interestingly, the amounts of various dipeptides were different in the one-year, five-year and thirty-year-old vinegars. Dipeptides include compounds that can affect the taste characteristics of foods and show pharmaceutical functions such as amelioration of colitis symptoms *via* participating in the inflammatory and immunomodulatory signaling pathways (Ge et al., 2021). The dipeptides reflected a combination of chemical and physical reactions during the aging process, for instance, L-cystathionine could be condensed by serine and homocysteine with cystathionine β-synthase (CBS) *via* the transsulfuration pathway (Jurkowska, Wróbel, Jasek-Gajda, & Rydz, 2022), and catalyzed to ammonia, pyruvate and L-homocysteine by cystathionine β-lyase (CBL) and pyridoxal 5′-phosphate (PLP) enzyme (Miyamoto, Katane, Saitoh, Sekine, & Homma, 2018), and thus the dipeptides might be used as biomarkers for differentiating the different-aged vinegars.

3.5. Changes in the organic acid contents

Organic acids such as lactic acid, citric acid, and 3-phenyllactic acid (PLA), in vinegar act as a buffer system that relieves the strong pungent smell and improves the taste of vinegar (Zhao et al., 2020). In our study, more than 60 acid-related metabolites were identified in one-year-old, five-year-old and thirty-year-old Yongchun *Monascus* vinegars (Table 3). Phenolic acids which can be produced by microbial catalysts during aging processes (Chen, Zhou, et al., 2016), including 3-cresotinic acid, protocatechuic acid and 3-hydroxyphenylacetic acid, correlated positively with the age of the vinegar (Table 3). In Kucha, a special tea in China, 3-cresotinic acid correlated positively with bitterness (Qin et al., 2020). Protocatechuic acid that belongs to hydroxybenzoic acids (HBAs) has shown various potential pharmacological functions, e.g., antibacterial, anti-inflammatory and antidiabetic activities, can be biosynthesized through shikimate pathway (Kim, Kim, Sim, & Ahn, 2020). 3-Hydroxyphenylacetic acid (3-HPAA) decreased blood pressure in a dose-dependent manner through vessel relaxation *in vivo* and exerted vasorelaxant effects *in vitro* (Dias, Pourová, Vopršalová, Nejmanová, & Mladěnka, 2022). Moreover, more than ten nitrogen-containing metabolites were identified (Table 3). Among the nitrogen-containing metabolites, the relative concentration of γ-Glu-Glu, a Kokumi γ-glutamyl-dipeptide (Lee et al., 2018), decreased with aging time. The relative concentration of isovaleric acid, a volatile, branched short-chain fatty acid (SCFA), exhibited different flavors and caused inhibition of smooth muscle cells in concentration-dependent manner (Blakeney, Crowe, Mahavadi, Murthy, & Grider, 2019; Coelho, Genisheva, Oliveira, Teixeira, & Domingues, 2017), was lowest in the five-year-old vinegar and highest in the thirty-year-old vinegar. In addition, the relative concentrations of SCFA derivatives such as 2-hydroxy-2-methylbutyric acid and levulinic acid increased with aging time and those of succinic acid semialdehyde and methyloxovaleric acid decreased. The relative concentration of 3-(4-hydroxyphenyl) propionic acid, known as desaminotyrosine (DAT) or phloretic acid, was higher in the five-year and thirty-year-old vinegars than in the one-year-old vinegar. Notably, 3-(4-hydroxyphenyl) propionic acid protected from influenza through improving the immune response (Steed et al., 2017). Overall, the results indicated that the organic acids that changed during the aging process could exert potential taste or pharmaceutical characteristics.

3.6. Numbers of changed metabolites and the corresponding pathway analysis

A total of 392 metabolites were in common, accounting for 48%, 45%, and 61% of the changed metabolites in comparing one-to-five year, one-to-thirty year and five-to-thirty year-old vinegars, respectively (Fig. 6A). In addition, 126, 84, and 54 metabolites, accounting for 15%, 10%, and 8% of the changed metabolites were unique to one-to-five year, one-to-thirty year, and five-to-thirty year-old vinegar comparisons, respectively. The main changes in the during the aging time were in beta-alanine metabolism, tyrosine metabolism, novobiocin biosynthesis, lysine biosynthesis, lysine degradation, and arginine and proline metabolism pathways (Fig. 6B), indicating that amino acid related pathways played important roles in the aging process. The results showed that not only the metabolite concentrations but also their

Table 3
Acid-related metabolites identified by CIL LC-MS.

NO.	Organic acids	HMDB	Relative ion intensity ($\times 10^3$)		
			YMV1	YMV5	YMV30
1	Isovanillic acid	HMDB60003	0.07 ± 0.02^{b}	0.04 ± 0.01^{b}	0.13 ± 0.02^{a}
2	γ-glutamylglutamic acid	HMDB11737	295.6 ± 48.39^{a}	124.42 ± 18.66^{b}	85.87 ± 11.82^{b}
3	3-Aminoisobutanoic acid	HMDB03911	403.64 ± 40.91^{b}	443.35 ± 30.47^{b}	1040.34 ± 17.88^{a}
4	4-Guanidinobutanoic acid	HMDB03464	133.28 ± 21.74^{b}	108.43 ± 21.47^{b}	224.42 ± 18.07^{a}
5	5-Aminopentanoic acid	HMDB03355	54,756.69 ± 3654.86^{c}	154,492.7 ± 12,187.37^{a}	122,562.83 ± 3681.87^{b}
6	3-Cresotinic acid	HMDB02390	9028.21 ± 506.86^{c}	11,134.23 ± 980.20^{b}	22,518.36 ± 793.40^{a}
7	2,4-Diaminobutyric acid	HMDB02362	55.18 ± 12.16^{b}	68.86 ± 23.12^{ab}	109.3 ± 21.01^{a}
8	3-(4-Hydroxyphenyl)propionic acid	HMDB02199	19.17 ± 7.22^{a}	22.48 ± 5.08^{a}	18.66 ± 11.07^{a}
9	*N*-Methyl-a-aminoisobutyric acid	HMDB02141	79.01 ± 10.07^{c}	218.82 ± 19.78^{b}	401.7 ± 104.05^{a}
10	Itaconic acid	HMDB02092	25.28 ± 4.62^{a}	23.11 ± 2.54^{a}	18.02 ± 2.00^{a}
11	Imidazoleacetic acid	HMDB02024	1685.26 ± 72.59^{b}	1483.52 ± 159.17^{b}	1909.97 ± 98.56^{a}
12	2-Hydroxy-2-methylbutyric acid	HMDB01987	4.34 ± 0.88^{b}	6.71 ± 1.09^{ab}	7.89 ± 2.73^{a}
13	2-Ethyl-2-hydroxybutyric acid	HMDB01975	16.7 ± 6.23^{a}	20.43 ± 5.29^{a}	18.14 ± 6.60^{a}
14	Salicylic acid	HMDB01895	0.08 ± 0.02^{a}	0.1 ± 0.01^{a}	0.08 ± 0.01^{a}
15	Benzoic acid	HMDB01870	0.87 ± 0.15^{b}	1.24 ± 0.14^{b}	2.63 ± 0.46^{a}
16	3,4-Dihydroxymandelic acid	HMDB01866	12.94 ± 2.06^{a}	22.22 ± 2.41^{a}	18.77 ± 11.2^{a}
17	Protocatechuic acid	HMDB01856	2740.22 ± 212.79^{b}	3001.7 ± 483.73^{b}	4351.87 ± 399.21^{a}
18	Glutaconic acid	HMDB01657	0.22 ± 0.01^{a}	0.22 ± 0.06^{a}	0.12 ± 0.01^{b}
19	2-Hydroxycaproic acid	HMDB01624	0.1 ± 0.02^{b}	0.14 ± 0.03^{ab}	0.16 ± 0.02^{a}
20	Diaminopimelic acid	HMDB01370	3332.72 ± 114.26^{a}	2282.84 ± 217.16^{b}	2345.04 ± 81.36^{b}
21	3,4-Dihydroxybenzeneacetic acid	HMDB01336	586.47 ± 151.88^{a}	549.72 ± 93.48^{a}	701.73 ± 207.88^{a}
22	Succinic acid semialdehyde	HMDB01259	0.56 ± 0.12^{a}	0.19 ± 0.03^{b}	0.13 ± 0.04^{b}
23	5-Aminolevulinic acid	HMDB01149	9553.65 ± 280.83^{a}	5754 ± 460.9^{b}	2810.43 ± 30.24^{c}
24	trans-Aconitic acid	HMDB00958	0.03 ± 0.00^{a}	0.02 ± 0.00^{a}	0.03 ± 0.01^{a}
25	Isoferulic acid	HMDB00955	16.45 ± 3.8^{c}	143.16 ± 17.73^{a}	81.46 ± 1.12^{b}
26	Monomethyl glutaric acid	HMDB00858	8.99 ± 0.57^{a}	1.5 ± 0.17^{b}	1.43 ± 0.19^{b}
27	Salicyluric acid	HMDB00840	10.85 ± 3.16^{c}	92.95 ± 17.4^{a}	37.92 ± 6.24^{b}
28	Phenyllactic acid	HMDB00779	0.19 ± 0.05^{b}	0.33 ± 0.05^{ab}	0.42 ± 0.10^{a}
29	Hydroxyphenyllactic acid	HMDB00755	0.07 ± 0.01^{c}	0.35 ± 0.04^{b}	0.44 ± 0.04^{a}
30	3-Hydroxymandelic acid	HMDB00750	697.41 ± 30.73^{b}	717.57 ± 38.95^{b}	1345.12 ± 268.21^{a}
31	Mesaconic acid	HMDB00749	0.01 ± 0.00^{b}	0.01 ± 0.00^{ab}	0.02 ± 0.00^{a}
32	Hydroxyisobutyric acid	HMDB00729	1.22 ± 0.23^{b}	2.61 ± 0.34^{a}	2.38 ± 0.3^{a}
33	Levulinic acid	HMDB00720	0.33 ± 0.05^{c}	0.46 ± 0.03^{b}	0.79 ± 0.08^{a}
34	Isovaleric acid	HMDB00718	8.67 ± 1.17^{b}	4.74 ± 1.19^{b}	14.61 ± 1.93^{a}
35	Hydroxypropionic acid	HMDB00700	17.98 ± 7.65^{a}	16.59 ± 1.49^{a}	28.06 ± 0.11^{a}
36	Methyloxovaleric acid	HMDB00695	1.2 ± 0.27^{b}	2.86 ± 0.4^{a}	3 ± 0.68^{a}
37	2-Hydroxyglutaric acid	HMDB00694	0.06 ± 0.01^{a}	0.02 ± 0.01^{a}	0.08 ± 0.09^{a}
38	Glutaric acid	HMDB00661	0.22 ± 0.03^{b}	0.59 ± 0.16^{b}	2.03 ± 0.48^{a}
39	Ethylmalonic acid	HMDB00622	4.91 ± 0.5^{b}	1.77 ± 0.22^{b}	8.82 ± 1.87^{a}
40	3-Methyladipic acid	HMDB00555	0.04 ± 0.02^{a}	0.09 ± 0.05^{a}	0.02 ± 0.02^{a}
41	Aminoadipic acid	HMDB00510	231.52 ± 43.89^{b}	267 ± 72.97^{b}	532.03 ± 7.47^{a}
42	4-Hydroxybenzoic acid	HMDB00500	24,378.86 ± 641.38^{b}	22,527.34 ± 1760.42^{b}	33,394.54 ± 1092.63^{a}
43	3-methyl-2-oxo-Valeric acid	HMDB00491	0.55 ± 0.06^{c}	1.35 ± 0.28^{b}	2.02 ± 0.28^{a}
44	Vanillic acid	HMDB00484	9.41 ± 2.24^{a}	1.83 ± 0.37^{b}	1.52 ± 0.02^{b}
45	Alpha-aminobutyric acid	HMDB00452	49,116.73 ± 1979.48^{c}	109,982.41 ± 9117.68^{b}	197,107.09 ± 11,969.93^{a}
46	3-Hydroxyphenylacetic acid	HMDB00440	9028.21 ± 506.86^{c}	11,134.23 ± 980.20^{b}	22,518.36 ± 793.40^{a}
47	Citramalic acid	HMDB00426	0.03 ± 0.00^{b}	0.16 ± 0.02^{a}	0.15 ± 0.01^{a}
48	3,4-Dihydroxyhydrocinnamic acid	HMDB00423	0.01 ± 0.00^{c}	0.02 ± 0.00^{b}	0.04 ± 0.01^{a}
49	2-Hydroxy-3-methylbutyric acid	HMDB00407	1.6 ± 0.71^{b}	1.85 ± 0.48^{b}	3.12 ± 0.23^{a}
50	2-Isopropylmalic acid	HMDB00402	0.06 ± 0.01 b	0.14 ± 0.02a	0.12 ± 0.02a
51	3-Hydroxymethylglutaric acid	HMDB00355	0.07 ± 0.02^{c}	0.25 ± 0.06^{b}	0.39 ± 0.01^{a}
52	Vanillylmandelic acid	HMDB00291	11.94 ± 2.52^{b}	72.91 ± 11.89^{a}	73.14 ± 4.03^{a}
53	Seryl-glutamic acid	HMDB0029038	1095.97 ± 83.51^{a}	423.64 ± 69.57^{b}	268.03 ± 38.3^{c}
54	Glutamyl-aspartic acid	HMDB0028815	445.56 ± 20.73^{a}	387.3 ± 28.17^{b}	349.16 ± 2.95^{c}
55	Arginyl-glutamic acid	HMDB0028708	30.83 ± 8.31^{a}	16 ± 4.86^{b}	11.42 ± 2.47^{b}
56	Alanyl-glutamic acid	HMDB0028686	3141.29 ± 222.93^{b}	2979.9 ± 315.06^{b}	4160.15 ± 72.74^{a}
57	Alanyl-aspartic acid	HMDB0028683	5395.22 ± 782.61^{a}	4754.58 ± 537.86^{a}	2959.91 ± 168.01^{b}
58	Pantothenic acid	HMDB00210	53.64 ± 5.37^{c}	98.27 ± 20.68^{b}	245.48 ± 32.39^{a}
59	Phenylacetic acid	HMDB00209	1.93 ± 1.61^{a}	0.44 ± 0.33^{a}	6.29 ± 6.83^{a}
60	Methylmalonic acid	HMDB00202	0.02 ± 0.00^{b}	0.05 ± 0.00^{a}	0.05 ± 0.01^{a}
61	Aspartic acid	HMDB00191	94,544.94 ± 5906.28^{c}	190,995.03 ± 19,337.61^{b}	297,168.56 ± 23,674.91^{a}
62	Lactic acid	HMDB00190	3.52 ± 1.24^{b}	3.94 ± 0.79^{b}	5.65 ± 0.38^{a}
63	Gentisic acid	HMDB00152	0.03 ± 0.01^{b}	0.05 ± 0.01^{a}	0.05 ± 0.00^{a}
64	Glutamic Acid	HMDB00148	947,962.43 ± 50,668.76^{a}	250,572.92 ± 23,744.89^{b}	201,843.19 ± 10,253.81^{b}
65	Homovanillic acid	HMDB00118	158.31 ± 2.68^{b}	165.49 ± 0.76^{b}	522.61 ± 56.27^{a}
66	γ-aminobutyric acid	HMDB00112	1,142,328.06 ± 57,517.03^{a}	933,530.88 ± 40,847.47^{b}	635,738.82 ± 21,906.24^{c}
67	Acetoacetic acid	HMDB00060	0.15 ± 0.04^{a}	0.14 ± 0.02^{a}	0.11 ± 0.01^{a}
68	Acetic acid	HMDB00042	23.64 ± 3.87^{a}	19.77 ± 2.68^{a}	24.41 ± 1.01^{a}
69	2-Ketobutyric acid	HMDB00005	0.03 ± 0.00^{a}	0.03 ± 0.01^{a}	0.02 ± 0.00^{b}

"Organic acids" were identified based on the accurate mass and retention time (tier 1 level). The different superscript letters within a row indicates a statistically significant difference at $p < 0.05$.

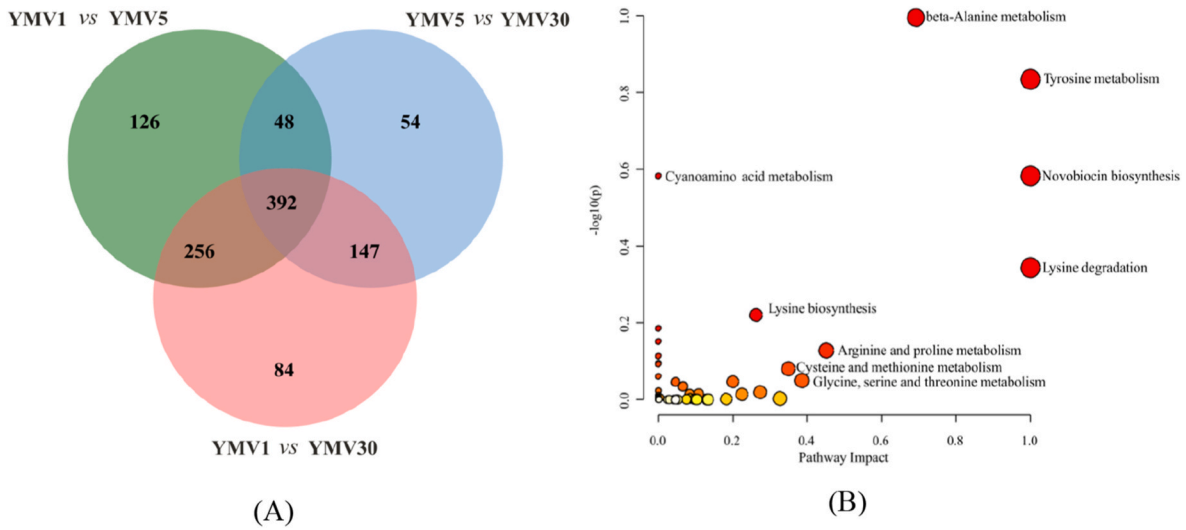

Fig. 6. Number of changed metabolites in Yongchun *Monascus* vinegars and the corresponding pathway analysis. A) Common and unique metabolites in one-to-five year, one-to-thirty year, and five-to-thirty-year comparisons. YMV1, one-year-old; YMV5, five-year-old; YMV30, thirty-year-old Yongchun *Monascus* vinegar. B) Pathway analysis based on the changed metabolites.

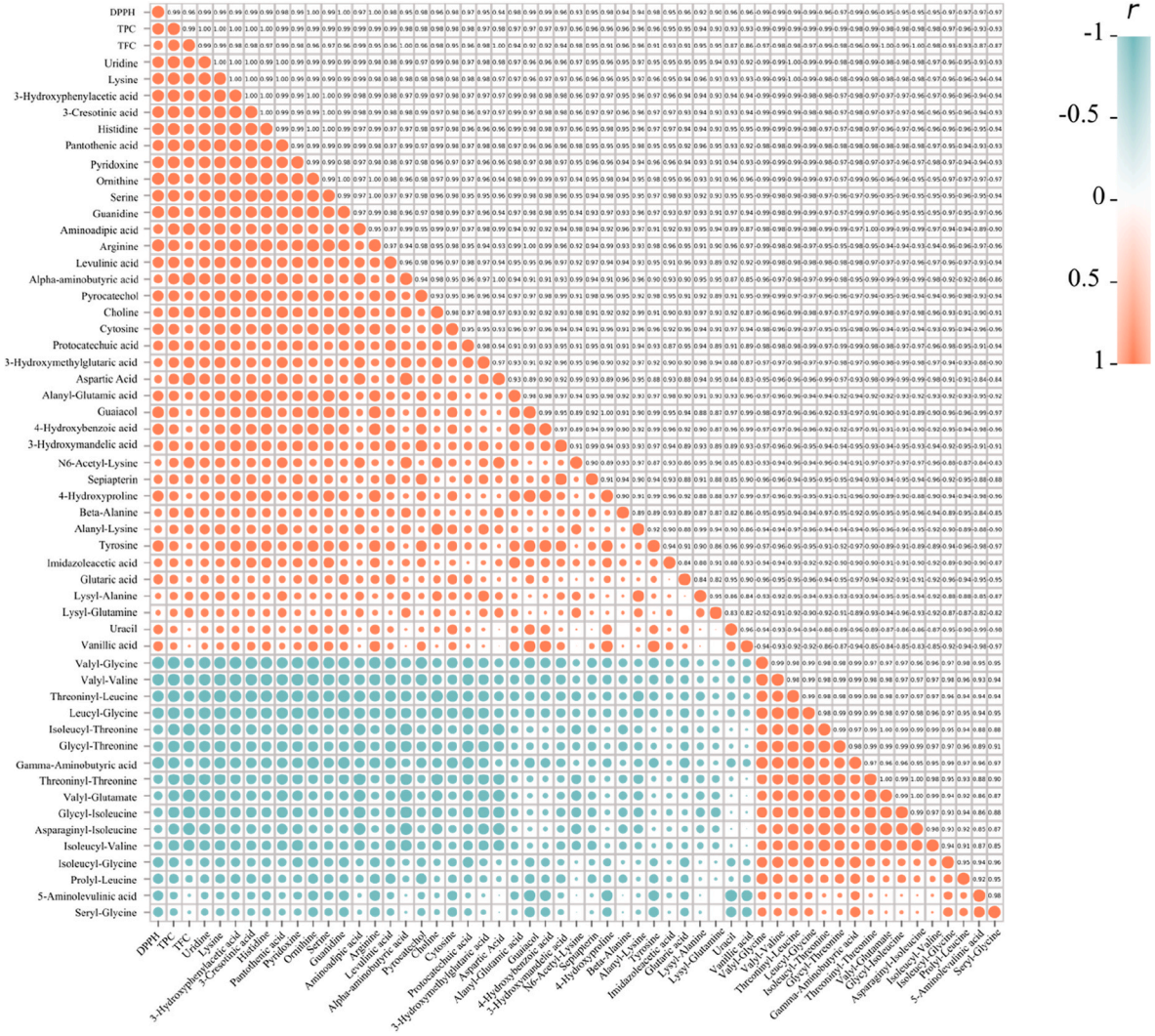

Fig. 7. Correlation analysis between the antioxidant activity of Yongchun *Monascus* vinegars and the detected metabolites.

compositions and metabolic pathways changed during the aging of Yongchun *Monascus* vinegar.

3.7. Correlation between antioxidant activity and metabolites

Correlation analysis indicated that the changes in metabolites were associated with the differences in antioxidant activity between the one-year-old, five-year-old and thirty-year-old Yongchun *Monascus* vinegars (Fig. 7, Table S4). The 36 metabolites that correlated positively with antioxidant activity included organic acids and free amino acids (Fig. 7). Among the positively correlating metabolites, the phenolic acids 3-hydroxyphenylacetic acid and 3-cresotinic acid are known to exhibit antioxidant activity. Organic acids were responsible for the antioxidant activities in fruit vinegars (Liu, Tang, Zhao, Gan, & Li, 2019). Amino acids like lysine, histidine and tyrosine have been regarded as antioxidants or acting as synergistic antioxidants due to their metal chelating activity and/or primary antioxidant reoxidation (Matsui et al., 2018). However, most of the dipeptides correlated negatively with antioxidant activities, suggesting that the amino acid sequence and structure of dipeptides played important roles in contributing to their antioxidant activity (Matsui et al., 2018).

4. Conclusions

We used HS-SPME-GC-MS and CIL LC-MS approaches combined with multivariate analysis to elaborate the metabolic differences between one-year-old, five-year-old and thirty-year-old Yongchun *Monascus* vinegars. A total of 1133 volatile and non-volatile metabolites changed significantly; 392 metabolites were in common whereas 126, 84, and 54 changed metabolites were unique to one-to-five year, one-to-thirty year, and five-to-thirty year-old vinegar comparisons, respectively. Amino acid related pathways played important roles in the aging process. Organic acids and dipeptides correlated with the antioxidant activity that increased with aging. The results suggested that organic acids and dipeptides could be used as biomarkers for differentiating the Yongchun *Monascus* vinegars of different age. The relationship between the sensory quality of different-aged YMVs and the differential metabolites needs further investigation. Moreover, revealing the dynamic mechanisms of the metabolites exerting potential taste or pharmaceutical characteristics need further attention. Taken together, the results not only reveal the age-related volatile compounds and non-volatile metabolites in Yongchun *Monascus* vinegar but also will provide useful knowledge for improving the quality of *Monascus* vinegar.

Funding

This work was supported by the National Key Research and Development Program of China (2021YFD1600804), Department of Science and Technology of Sichuan Province (2021JDJQ0038, 2022YFS0594), and Funds of Sichuan Academy of Agricultural Sciences (2019LJRCC033, 2019QYXK013 and 2021XKJS064).

CRediT authorship contribution statement

Ling Dong: Investigation, Data curation, Methodology, Writing, and Review. **Chi Zhao:** Investigation, Data curation, Methodology, Writing, and Review. **Fengju Zhang:** Investigation, Data curation, Methodology, Writing, and Review. **YingLun Ma:** Resources. **Chuan Song:** Investigation, Data curation, Methodology, Writing, and Review. **Petri Penttinen:** Writing, and Review. **Suyi Zhang:** Conceptualization, Supervision, and Review. **Zhihua Li:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The author declares no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

We thank Nova Medical Testing Inc.(Edmonton, AB, Canada, www.novamt.com) for providing the high performance chemical isotope labeling LC-MS experiment.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114169.

Appendix A. Supplementary data

Supplementary data related to this article can be found online.

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