

Untargeted GC–Orbitrap–HRMS fingerprinting reveals tree age-dependent changes in flavor-related metabolites of Musang King durian

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Abstract

BACKGROUND: Tree age is an important preharvest factor that can influence the chemical composition and flavor attributes of fruit, yet its effect on Musang King durian (MKD) quality remains poorly understood. In this study, an untargeted metabolomics approach using gas chromatography–high-resolution mass spectrometry (GC–Orbitrap–HRMS) was employed to comprehensively profile both volatile and non-volatile metabolites in MKD fruits harvested from young (5–9 years), middle-aged (10–15 years), and old (>16 years) trees.

RESULTS: Multivariate analysis revealed age-dependent flavor metabolite differentiation, with principal component analysis showing distinct clustering among tree age groups, and confirmed by partial least squares discriminant analysis. Key discriminant metabolites included palatinose, α -L-galactofuranoside, levoglucosan, L-(+)-rhamnopyranose, pyroglutamic acid, galactaric acid, 8-methylnonanoic acid, d-gluconic acid, *s*-isopentyl propanethioate, propane-1-thiol, and propyl-1-(propylthio)ethyl disulfide. Integrative analysis further showed that fruits from old trees contained the highest total sugars, middle-aged trees had the highest total organic acids, fatty acids, and amino acids, while young trees produced fruits rich in volatiles such as sulfur compounds, esters, aldehydes, and alcohols.

CONCLUSION: These findings highlight that tree age influences the metabolite composition of MKD fruits. These metabolic differences demonstrate the potential of metabolite fingerprinting for distinguishing MKD fruits from different tree ages and supporting strategies for quality authentication.

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Keywords: durian; flavor; fruit quality; non-volatile metabolites; volatile metabolites

INTRODUCTION

Durian (*Durio zibethinus* Murr), also known as the ‘king of fruits’, is a tropical fruit extensively grown in Southeast Asian countries, particularly in Malaysia, Thailand, Indonesia, and the Philippines.¹ It belongs to the family Malvaceae and genus *Durio*, with over 300 varieties across the regions.² Among varieties, Musang King durian (MKD), commonly regarded as the ‘king of durian’ in Malaysia, is especially popular in both local and international markets due to its superior texture and flavor.³ MKD possesses a strong aroma, slightly bitter taste, and thick, buttery, golden yellow flesh,⁴ attributed to fats, sugars, carotenoids, and volatile compounds (VOCs).^{5,6}

The flavor profile of a fruit determines its eating quality and its marketability.^{7,8} It results from the interaction between volatile and non-volatile metabolites,^{9,10} including sugars, organic acids, amino acids, fatty acids, and VOCs, which influence the flavor profile of the fruit.^{10–12} In durian fruits, VOCs such as volatile esters, alcohols, ketones, and sulfur compounds contribute to their characteristic aroma,^{13,14} which enhances marketability.¹⁵

These VOCs are products of fatty acid and amino acid metabolism.^{16–19} Additionally, nonvolatile compounds such as sugars, organic acids, and fatty acids play a central role in defining the fruit's taste.^{11,16,20,21}

The biosynthesis and accumulation of these compounds can be influenced by various preharvest factors, including genetic background,²² tree age,²³ and environmental conditions during growth.²⁴ Among these, tree age was reported to affect the fruit quality of many perennial crops through changes in physiological and metabolic processes.²⁵ For instance, it affected key

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physicochemical properties of fruits, such as pH, titratable acidity, soluble solids content, and total sugars,^{23,26–29} which define the fruit's flavor.^{8,30} While these findings provide evidence of quality changes resulting from tree age, they do not fully uncover how tree age affects fruit quality at the endogenous metabolite level, which is critical for explaining these changes in detail.

As trees grow and mature, physiological processes including mineral accumulation, allocation, and shift in metabolic activities influence the fruit's composition.^{27,31–33} These physiological changes can significantly impact the biosynthesis and accumulation of flavor-related metabolites in fruits. For instance, ethylene has been shown to regulate malic acid and citric acid biosynthesis in tomato fruits.³⁴ Similarly, tree age-dependent differences in sugar and organic acid profiles have been observed in jujube (*Ziziphus jujuba* Mill.) fruits.²³ In addition, nutrient accumulation, especially potassium, enhances sugar accumulation by promoting enzyme activities and gene expression involved in sucrose metabolism, thereby improving fruit flavor.³⁵

Various advanced instruments have been used to separate, analyze, and identify non-volatile and volatile compounds in durian pulp. However, due to the low concentration of key metabolites, more sensitive techniques like gas chromatography–high-resolution mass spectrometry (GC–Orbitrap-HRMS) have been introduced to improve mass accuracy and detection capabilities.³⁶ Although numerous studies have investigated durian flavor chemistry, these studies are largely focused on only volatile metabolites.^{11–14,16,37–41} Moreover, the impact of tree age on durian fruit quality has primarily been examined through physicochemical parameters,⁴² with limited understanding at the metabolite level.

To the best of our knowledge, the study reported here is the first to comprehensively profile both volatile and non-volatile metabolite profiles of MKD in relation to tree age using untargeted metabolomics with GC–Orbitrap-HRMS. In addition, chemometric fingerprinting approaches for discriminating fruit quality based on tree age remain unexplored. Therefore, the study aimed to investigate how tree age influences both non-volatile and volatile metabolites in MKD using an untargeted fingerprinting approach with GC–Orbitrap-HRMS. By identifying age-specific quality markers, the study not only enhances the understanding of flavor development in durian but also provides valuable tools for MKD differentiation and improved marketing strategies.

MATERIALS AND METHODS

Plant materials

Fresh, fully ripe MKD fruits from three tree age groups (5–9 years (young), 10–15 years (middle age), and >16 years (old)) were harvested from a commercial orchard in Raub (latitude 3° 03' N, longitude 101° 51' E), Pahang, Malaysia, during the peak season in November 2022. The durians were transported within 15 min to the processing facility at Hernan Corporation Sdn. Bhd. for selection and sorting. Only fruits with no visual defects were selected, packed in a plastic crate (70 cm × 50 cm × 25 cm), and transported to our laboratory using a reefer van. Six trees were selected from each tree age group (young, middle, and old), with one fruit collected from each tree, providing six biological replicates per tree age group (*N* = 18). The pulp from all locules of the durian fruits were pooled together to form a single composite sample of each biological replicate. Next, the part of the composite sample was flash-frozen in liquid nitrogen, packed in a polyethylene plastic bag (0.06 mm), and stored at –80 °C for VOC analysis.

The remaining portion of the composite sample was lyophilized (LaboGene Scanvac CoolSafe 4 L, UK) for 48 h, ground into fine powder using a grinder (IKA® A11 basic, Germany), tightly packed in a polyethylene plastic bag, and then stored at –80 °C for non-volatile metabolite composition analysis.

Untargeted nonvolatile metabolite profiling

Non-volatile metabolite extraction

Freeze-dried MKD pulp powder was extracted following the method proposed by Lisec *et al.*,⁴³ with modifications. First, 30 mg of lyophilized powdered sample was extracted with 1.4 mL of precooled methanol (99.8%), 1.5 mL of ultrapure water (4 °C), and 750 µL of chloroform (99.8%). Then, 70 µL of ribitol (0.2 mg mL^{–1} in ultrapure water) was added and vortexed for 10 s. Then, the mixture was incubated in a thermomixer at 60 °C and 550 rpm for 10 min and centrifuged for 15 min at 12 000 × *g*. Next, the supernatant was collected and run through a centrifuge evaporator (Genevac EZ-2 Elite, Sysmex Belgium NV) to remove solvents. Then, the extract was lyophilized overnight until dry. The lyophilized extract was then derivatized by the addition of 150 µL of methoximation reagent (20 mg mL^{–1} methoxyamine hydrochloride in pyridine) and stirred for 2 h at 50 °C. Then, 120 µL of methyl-*N*-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, St Louis, MO, USA) was added and stirred for 1 h at 50 °C. The mixture was transferred to GC vials with 250 µL inserts and immediately injected into the GC instrument.

GC–HRMS analysis for non-volatile metabolites

The metabolite profile of MKD pulp was analyzed using GC coupled with a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) according to Lisec *et al.*,⁴³ with modifications. The derivatized sample (1 µL) was injected at 280 °C in a split mode (1:50) using helium as the carrier gas at 1 mL min^{–1} flow and linear velocity as the flow control mode. The capillary column used was a TG-5SilMS (30 m × 0.25 mm × 0.25 µm; Thermo Scientific, USA) with column oven temperature programming starting at 70 °C for 2.5 min, increased to 350 °C at a rate of 50 °C min^{–1} and held for 4.5 min. MS parameters included an ion source and interface set at 300 °C, with an acquisition range of 50–700 *m/z* at resolution of 60 000 full width at half maximum (FWHM). The data were acquired and processed using Xcalibur software and TraceFinder general software (v.5.2 SP1) (Thermo Fisher Scientific). The identification of metabolites was confirmed by comparing acquired mass spectra and retention time with NIST17 (version 2.3, released at 2017) database entries. The relative concentrations of nonvolatile metabolites were estimated by the ratio of their peak area to the peak area of the internal standard (1 mg L^{–1} ribitol).

Untargeted volatile compound profiling

The extraction and analysis of VOCs were performed using solid-phase microextraction and a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) respectively, following the method of Sospeter *et al.*⁴⁰

Fatty acid profiling

Fat extraction

Fat extraction from the MKD fruit was done according to Nazari *et al.*,⁴⁴ with modifications. An amount of 300 mg of freeze-dried sample was homogenized with 2 mL of isopropanol containing 0.5% butylated hydroxytoluene. The mixture was incubated at 70 °C for 10 min in a thermomixer (Eppendorf AG, 22331, Hamburg). After cooling, 1 mL of chloroform, methanol (from Sigma-Aldrich, St Louis, MO, USA), and Milli-Q water were added to the

mixture. The mixture was vortexed and shaken at 750 rpm for 30 min at 60 °C. The homogenate was then cooled and centrifuged at $12\,000 \times g$ for 2 min. The supernatant was collected, and the remaining residue was extracted with an additional 1 mL each of chloroform and Milli-Q water. The mixture was shaken again at 750 rpm for 15 min at 60 °C. The resulting supernatants from both extractions were combined and centrifuged to separate the polar and nonpolar phases. The nonpolar phase (chloroform phase) was collected and dried using a centrifugal evaporator (Genevac EZ-2 Elite, Sysmex Belgium NV). To derivatize the dried extract, 100 mg of the sample was dissolved in 10 mL of hexane and reacted with 100 μ L of 2 N potassium hydroxide in methanol. The derivatized lipids were transferred to GC vials for GC analysis.

Determination of fatty acids by GC–HRMS

The fatty acid profile of durian pulp was analyzed according to Salleh *et al.*,⁴⁵ with modification, using a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The inlet temperature was maintained at 250 °C, with an injection volume of 1 μ L and a split ratio of 1:50. Hydrogen was used as the carrier gas at a constant head pressure of 53 kPa, corresponding to a linear velocity of 36 cm s^{−1} at 50 °C. The oven temperature started at 50 °C, held for 1 min, followed by a ramp of 25 °C min^{−1} to 200 °C, and then a ramp of 3 °C min^{−1} to a final temperature of 230 °C and held for 18 min. The detector temperature was set at 280 °C, with hydrogen supplied at 40 mL min^{−1}, air at 450 mL min^{−1}, and helium makeup gas at 30 mL min^{−1}. The resolving power was 120 000 FWHM. The acquisition scan range was set to 50–600 *m/z*.

Data analysis

In this study, six biological replicates were used. The collected fatty acid composition data were analyzed using analysis of variance (ANOVA) with JMP software version 17 Pro (SAS Institute, Inc., Cary, NC, USA) with means comparison via Tukey's HSD test ($P < 0.05$).

GC–MS data for nonvolatile and volatile metabolites were analyzed through preprocessing, multivariate analysis, and biomarker identification. Background signals were subtracted to remove noise and baseline shifts. Metabolites were identified based on a library match score $> 85\%$, and peak areas were normalized to internal standards. In accordance with Metabolomics Standards Initiative (MSI) guidelines,⁴⁶ metabolites were identified through spectral library matching without authentic reference standards confirmation were categorized as MSI level 2. Data were log-transformed (base 10) using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca>). Principal component analysis (PCA) was used for outlier detection and pattern recognition, while hierarchical cluster analysis applied Euclidean distance and Ward's method. Partial least squares discriminant analysis (PLS-DA) compared tree age effects on metabolite profiles, validated via cross-validation model to assess robustness using R^2 and Q^2 . Metabolites with a variable importance in projection (VIP) score ≥ 1.0 were identified as key discriminant markers. Subsequently, the influential nonvolatile and volatile metabolite markers underwent ANOVA, followed by *post hoc* Tukey's HSD test ($P < 0.05$) using JMP Pro 17 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Non-volatile metabolite profiling

GC–Orbitrap HRMS detected a total of 64 metabolites in MKD fruit pulp, including sugar and sugar derivatives, amino acids, organic acids and derivatives, and other compounds. The total relative

concentration of sugars did not differ significantly among tree ages. However, significant differences were observed in the relative concentration of organic acids and amino acids, which were higher in fruits from middle-aged and old-aged trees compared to those from young trees (Fig. 1).

To examine differences in the metabolite profiles of MKD from different tree ages (young, middle, and old), the chromatographic peak areas were analyzed using unsupervised PCA. The PCA score plot explains a cumulative variation of 70.8%. This indicates that more than half of the variability in nonvolatile metabolite data was captured by principal components 1 and 2 (Fig. 2(a)). A distinct, clear separation of durian from different tree ages was observed in the PCA score plot. This suggests that variations in tree age significantly influence the metabolite profiles of MKD fruit (Fig. 2(a)).

In addition, a heatmap and dendrogram were used as a tool to further visualize the similarities of the samples and the metabolites, which drive their relationships and the concentration of compounds in samples in different tree ages (Fig. 2(b)). Samples were clustered into two major clusters. Cluster one comprised samples from young and old trees, and cluster two comprised samples from middle-age trees. For instance, the key metabolites that drive clustering of young and old trees were pipercolonic acid, L-asparagine, 5-aminolevulinic acid, GABA, acetic acid, D,L-tyrosine, hypotaurine, and 2-aminoisobutyric acid (highlighted by green rectangle). These metabolites were down-regulated in the fruits from young and old trees (Fig. 1(b)). The clustering of young and old tree fruits together, while middle-aged tree fruits formed a separate cluster, reflects that the metabolic profiles of fruits from young and old trees are comparable to those from middle-aged trees (Fig. 2(b)).

Additionally, PLS-DA was employed as a supervised technique to examine the key non-volatile metabolites driving the differences between the three tree age groups. PLS-DA reveals distinct, clear clusters separating young, middle, and old tree ages with a cumulative variation of 69.6%, explained by principal components 1 and 2 (Fig. 3(a)). Both R^2 and Q^2 in cross-validation model exceeded 0.7, implying that the PLS-DA model is robust (Fig. 3(c)). Furthermore PLS-DA revealed the top 15 influential metabolites, which were selected based on $VIP \geq 1$ and the heatmaps showing the relative abundance of key nonvolatile metabolites in different age groups including sugars, organic acids, and nucleoside (Fig. 3(b)). However, our focus in this study was entirely on metabolites that potentially influence the flavor perception of durian fruits.

Changes of key sugars of MKD from trees of different ages

Fructose isomer and palatinose were higher in durian from young trees. α -L-Galactofuranoside, levoglucosan, and L-(+)-rhamnopyranose were higher in fruits from old trees, and this content decreased as the tree age increased while mannose-6-phosphate was prevalent in fruits from the middle-aged trees (Fig. 4).

Changes of key organic acids of MKD from trees of different ages

A total of four organic acids were identified as key markers differentiating MKD durian from different tree ages ($VIP \geq 1$), including pyroglutamic acid, galactaric acid, 8-methylnonanoic acid, and D-gluconic acid (Fig. 4).

Volatile metabolite profiling

In this study, GC–Orbitrap MS revealed 69 volatile compounds in the headspace fraction of the MKD pulp from different tree ages.

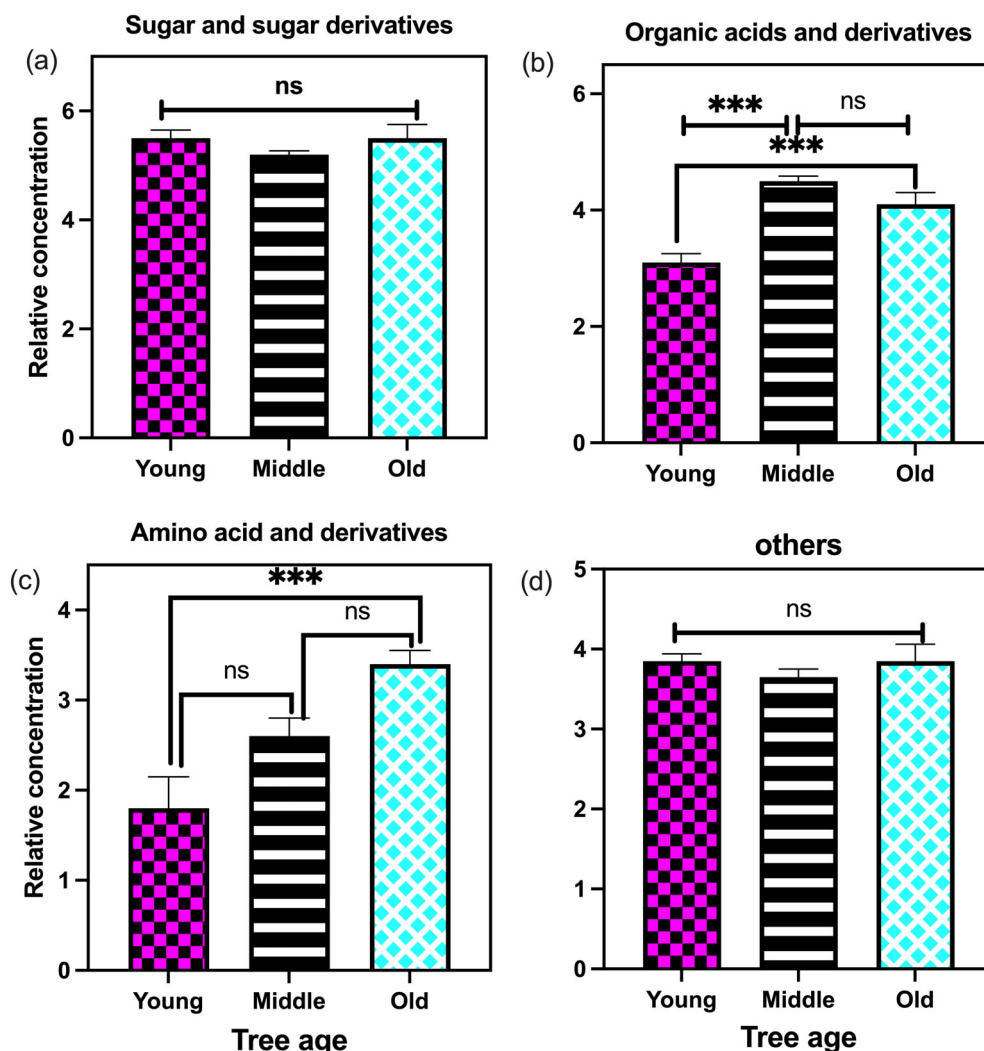


Figure 1. Relative concentrations of non-volatile metabolites of MKD fruit in different tree ages (young, middle, and old). Means and standard error were used to present each nonvolatile metabolite chemical group. Symbol (*) means a significant difference between groups with Tukey's HSD test ($P < 0.05$). *** $P < 0.001$; Y-axis, relative concentration; X-axis, tree age.

Based on their chemical structure, these compounds can be grouped into various categories: esters, sulfur compounds, alcohols, aldehydes, ketones, aromatic hydrocarbons, alkanes, alkenes, and others in the durian VOC profile. Figure 5 visualizes the total relative concentration of volatile metabolites in MKD as affected by tree age. Total sulfur compounds were significantly higher in fruits from young trees compared to fruits from middle-aged trees, and no significant differences were observed in sulfur concentration between MKD fruits from young and old trees ($P < 0.05$) (Fig. 5(a)). Ester and alcohol compounds were higher in fruits from young trees compared to fruits from middle and old trees, whereas aldehydes and ketones showed no significant differences across all tree age groups (Fig. 5(b)–(e)). Additionally, terpenes were significantly higher in fruits from young and old-age trees compared to those from middle-aged trees ($P < 0.05$) (Fig. 5(f)).

Similarly, Fig. 6(a) presents a PCA scatter plot of volatile compound clusters from different tree age groups; principal components 1 and 2 capture the cumulative variation of 64.7%. This reflects that the first two components capture significant variations in durian fruits from different tree age groups. Furthermore,

the proximity between the samples on the scatter plot shows comparable behavior of VOC profiles. Furthermore, a heatmap and dendrogram visualized the similarities of the samples and the VOCs, which drive their relationships and the concentration of compounds in samples in different tree ages (Fig. 6(b)). There are some similarities shown between samples of the fruits from old trees and young trees. While fruits from middle-age trees showed a profile distinct from that of young and old trees (Fig. 6(b)).

The PLS-DA model was used to investigate the key volatile compounds that drive the differences between durian fruits from varying tree ages. Performance of the PLS-DA model exhibited goodness-of-fit and predictive ability, with both R^2 and Q^2 values exceeding 0.7 (Fig. 7(c)). Figure 7(a) depicts the PLS-DA model of the six biological replicates of durians from each tree age group (young, middle, and old). Samples from young, middle, and old trees were distinctly separated. This suggests that the tree age strongly influences the volatile compound profile of MKD fruits. The 15 top volatile compounds driving the differences in MKD fruits between the three tree age groups were obtained based on VIP from the PLS-DA model ($VIP \geq 1$) (Fig. 7(b)). The heatmaps

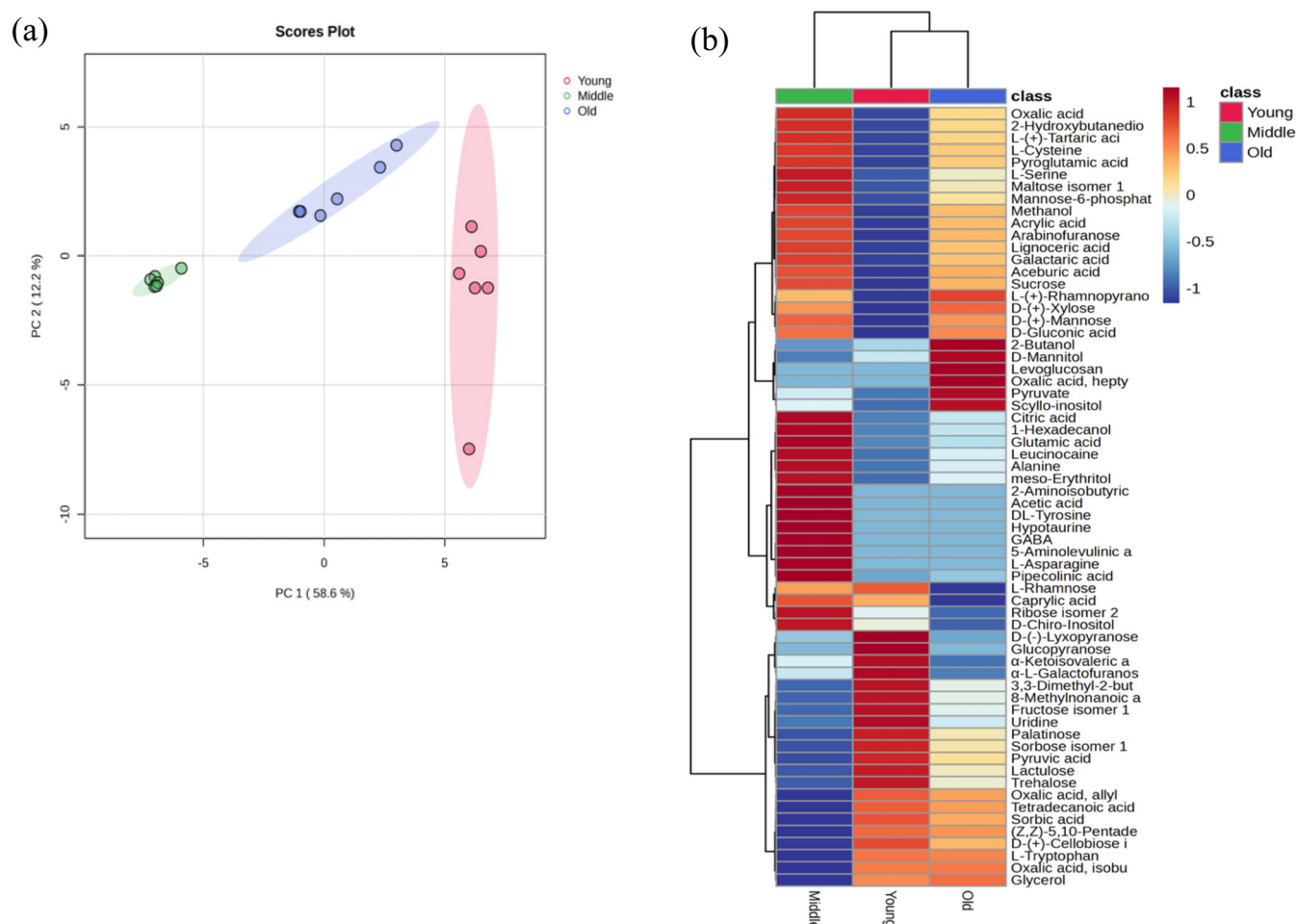


Figure 2. (a) PCA scatter plot showing the clustering of nonvolatile metabolites in MKD from three tree age groups (young, middle, and old), with the first two principal components explaining a cumulative variance of 70.8%. (b) Heatmap illustrating the relative concentration patterns of metabolites across the three tree age groups. Nonvolatile metabolites are shown in columns and their concentration in the colored cell; the brown colored cell reflects the high concentration of the compound, and blue indicates low compound concentration.

on the right-hand side show the relative abundance of key volatile metabolites in different age groups (Fig. 7(b)).

Changes of key sulfur volatile compounds of MKD fruits from trees of different ages

Sulfur compounds were the most abundant compounds after esters in MKD durian pulp (about 23–24%). Propane-1-thiol and propyl-1-(propylthio)ethyl disulfide were upregulated in fruits from young trees (Fig. 8(a),(d)), while 3-(ethylthio)propanoic acid was higher in fruits from old trees (Fig. 8(e)).

Changes of key esters, ketones, alcohols, and alkenes of MKD fruits from trees of different ages

(Z)-Oct-5-en-1-ol, 4-ethenyl-1,2-dimethylbenzene, 1-ethyl-3,5-dimethylbenzene, and 1-hydroxybutan-2-one were higher in fruits from young trees (Fig. 8). While isopropylbenzene and methyl (E)-2-methyl-2-butenate was higher in fruits from old trees (Fig. 8).

Fatty acid composition of MKD from trees of different ages

The relative concentrations of lipid methyl esters in MKD fruits from young, middle, and old trees are presented in Fig. 9(a)–(g). Methyl palmitoleate, butyric acid methyl ester and tetradecanoic acid, 10,13-dimethyl-, methyl ester were significantly lower in

fruits from old trees than in those from middle-aged trees, while no differences were observed between young and middle-aged trees (Fig. 9(a),(d),(f)) ($P < 0.05$). Similarly, the relative concentration of hexadecanoic acid methyl ester, methyl tetradecanoate, butyric acid methyl ester and elaidic acid methyl ester remained unchanged across tree ages (Fig. 9(b),(c),(e),(g)).

Integrative analysis of metabolite composition of MKD fruit for optimum tree age selection

The PCA biplot revealed that MKD fruits from different tree ages showed distinct quality attributes based on their metabolite composition (Fig. 10(a)). The total relative concentration of sulfur compounds, esters, terpenes, alcohols, and aldehydes was more associated with fruits from young trees. Similarly, fruits from older trees were associated with higher sugar content. Meanwhile, fruits from middle-aged trees exhibited higher levels of fatty acids, organic acids, and amino acids (Fig. 10(b)).

DISCUSSION

Tree age is an important factor influencing the metabolite composition of MKD fruit, demonstrating its role as one of the determinants of the biochemical quality of the fruit. The findings of this study revealed that tree age significantly affected the nonvolatile

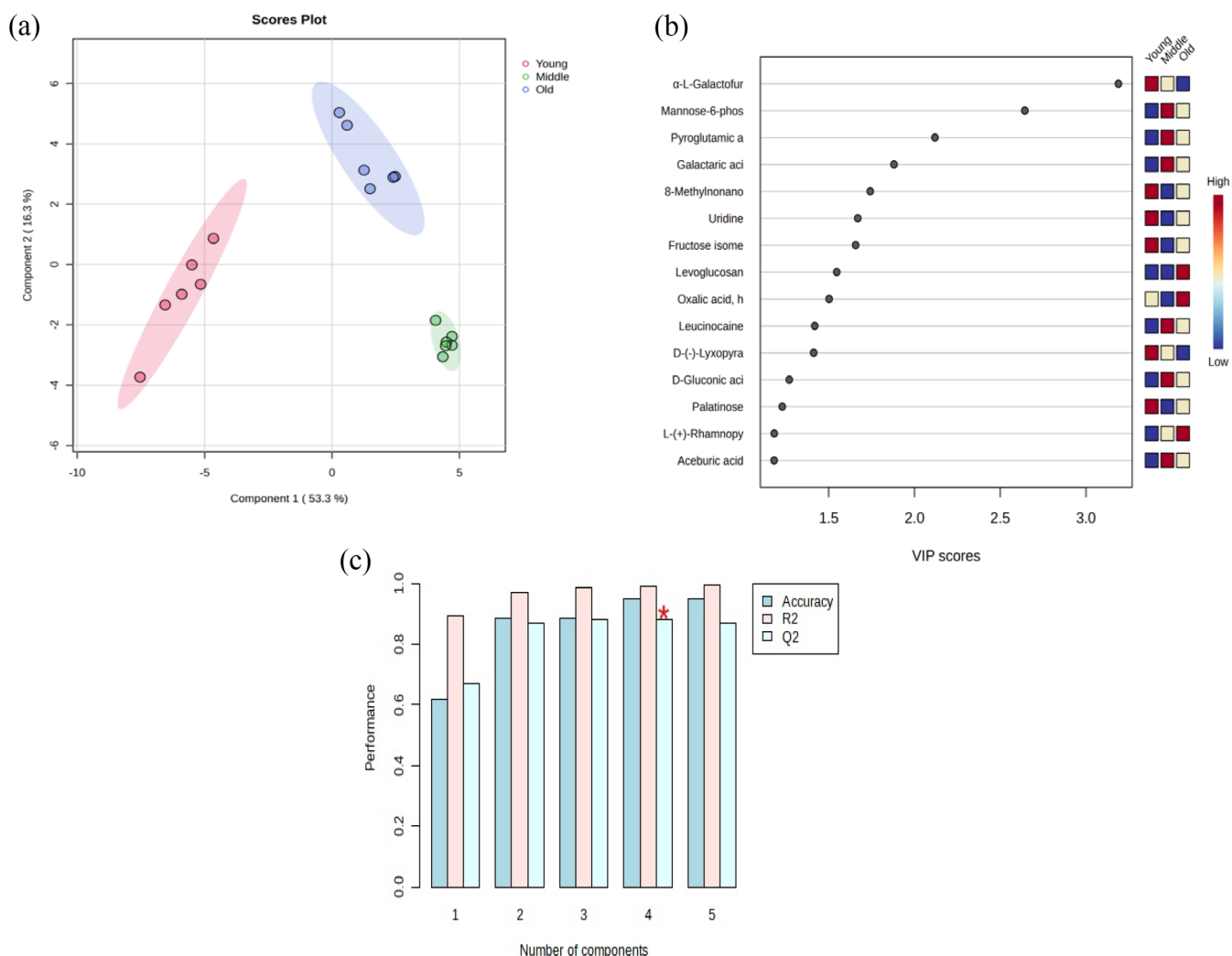


Figure 3. (a) PLS-DA of non-volatile metabolites in MKD from young-, middle-, and old-aged trees ($n = 6$). The PLS-DA plots display the variance in the predictive components. Component 1 (53.3%) and component 2 (16.3%) display distinct clusters among durians of different tree ages. (b) VIP displays important markers prevalent to distinguish durian from different tree ages. (c) Cross-validation parameter plots displaying R^2 and Q^2 of the PLS-DA model of MKD across different tree ages.

and volatile metabolite compositions of MKD fruit, suggesting that the developmental age modulates metabolic pathways linked to flavor-related metabolites. Similar, age-dependent changes in fruit metabolite composition, especially sugars and organic acids, have also been reported in other fruit species.²³

Among non-volatile metabolites, tree age affected carbohydrate metabolism. Fruits from young trees had higher levels of fructose, whereas L-rhamnopyranose was high in fruits from old trees and mannose-6-phosphate together with D-gluconic acid were high in fruits from middle-aged trees. These metabolites participate in different branches of the carbohydrate metabolic pathways, so their differential accumulation indicates that tree age influences carbohydrate metabolic networks. Fructose is one of the major soluble sugar contributing to the sweetness of fruits,⁴⁷ and it is regulated through carbohydrate synthesis, transport, and degradation pathways, which involve sugar transporters and glycosidases.⁴⁸ The accumulation of fructose in young trees reflects the increased carbohydrate metabolism associated with the higher photosynthetic activities commonly reported for younger perennial trees.⁴⁹ Arguably, the high level of mannose-6-phosphate in middle-aged trees indicates higher flux through glycolysis and the

pentose phosphate pathway, supporting GDP-mannose biosynthesis required for cell wall polysaccharide and glycoprotein synthesis.^{50–52} Similarly, the accumulation of L-rhamnopyranose in fruit from old trees suggests an age-related alteration in deoxy sugar metabolism. L-Rhamnopyranose has been associated with sweetness in some fruit species,⁵³ although we cannot rule out its contribution to the sensory properties of MKD because sensory evaluation was not assessed in this study.

Tree age also influenced the volatile metabolites of MKD fruits. Fruits from young trees were observed to have higher levels of sulfur compounds, esters, alcohols, and aldehydes, whereas several sulfur-containing and ester compounds, such as methyl (*E*)-2-methyl-2-butenate and 3-(ethylthio)propanoic acid, were more abundant in fruits from old trees. Sulfur compounds contribute to the distinct aroma of durian fruits⁵⁴ and are often associated with the methionine-derived pathway, in which methionine gamma-lyase contributes to the formation of sulfur volatiles from methionine precursors.¹⁸ The differential sulfur compounds observed in different tree age groups may reflect the changes in sulfur metabolism, which might be linked with tree ageing. However, there is a lack of studies explaining the age-related mechanism behind volatile compound pathway

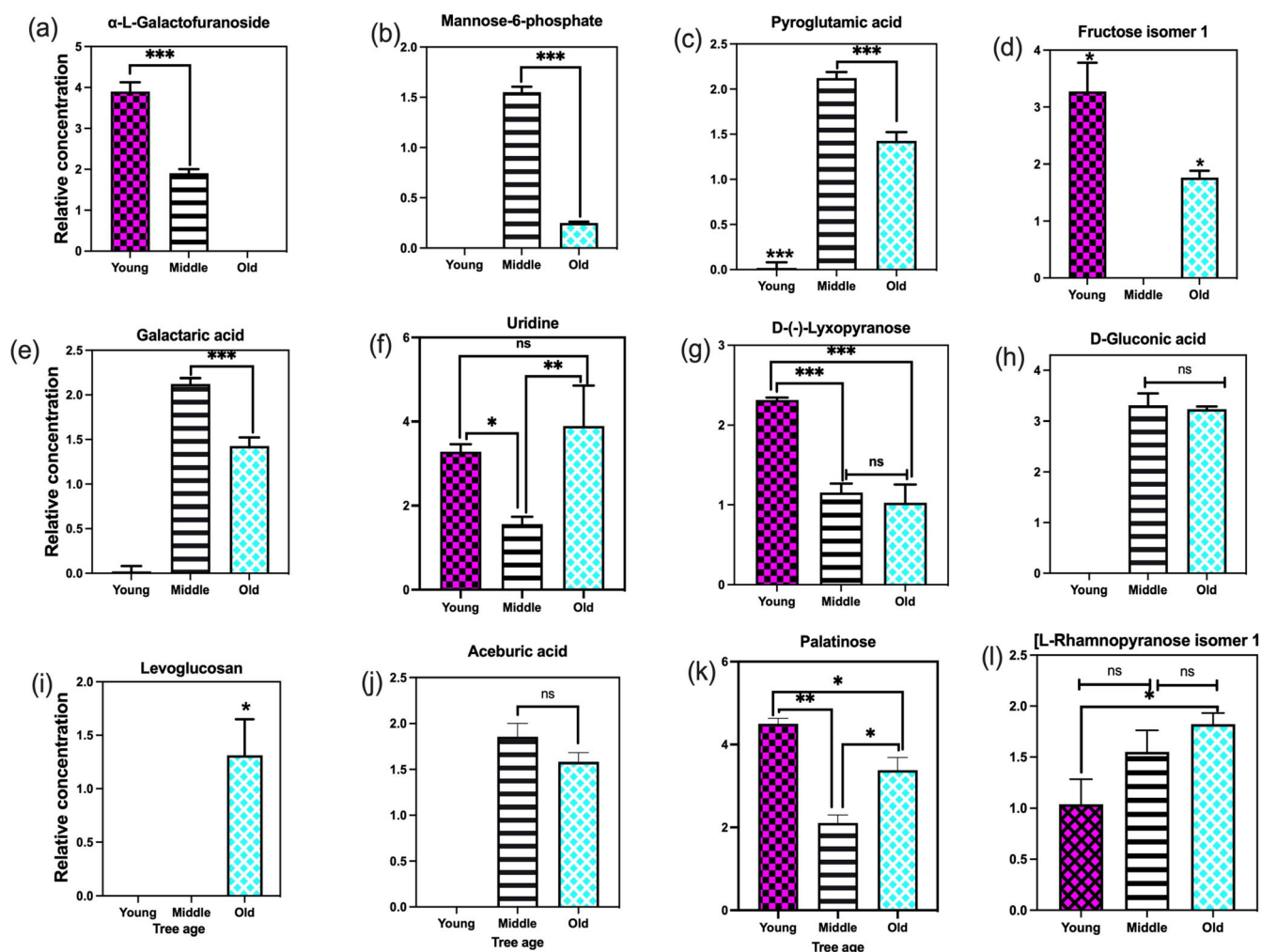


Figure 4. Changes in influential metabolite markers in MKD as affected by tree age (young, middle, and old). Symbol (*) means significantly different between groups with Tukey's HSD test ($P < 0.05$). Y-axis, relative concentration.

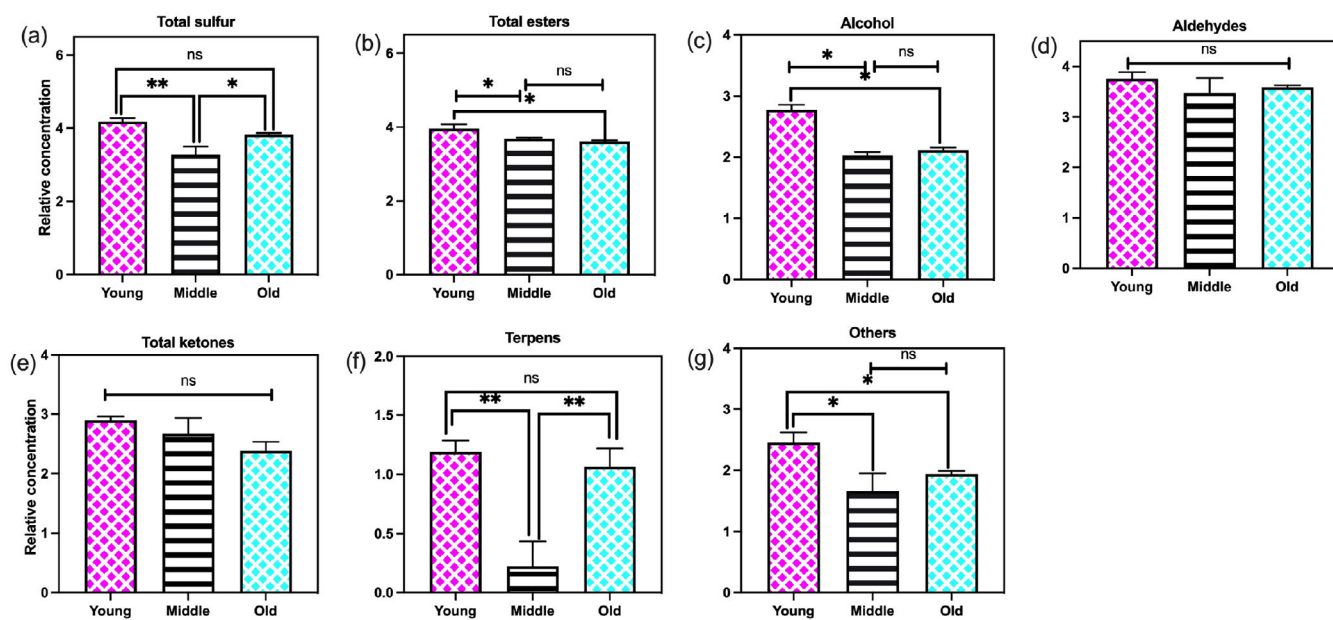


Figure 5. Relative concentrations of volatile metabolites of MKD fruit from different tree ages (young, middle, and old). The means and standard error were used to present each volatile metabolite's chemical group. Symbol (*) means significantly different between groups with Tukey's HSD test; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Y-axis, relative concentration; X-axis, tree age.

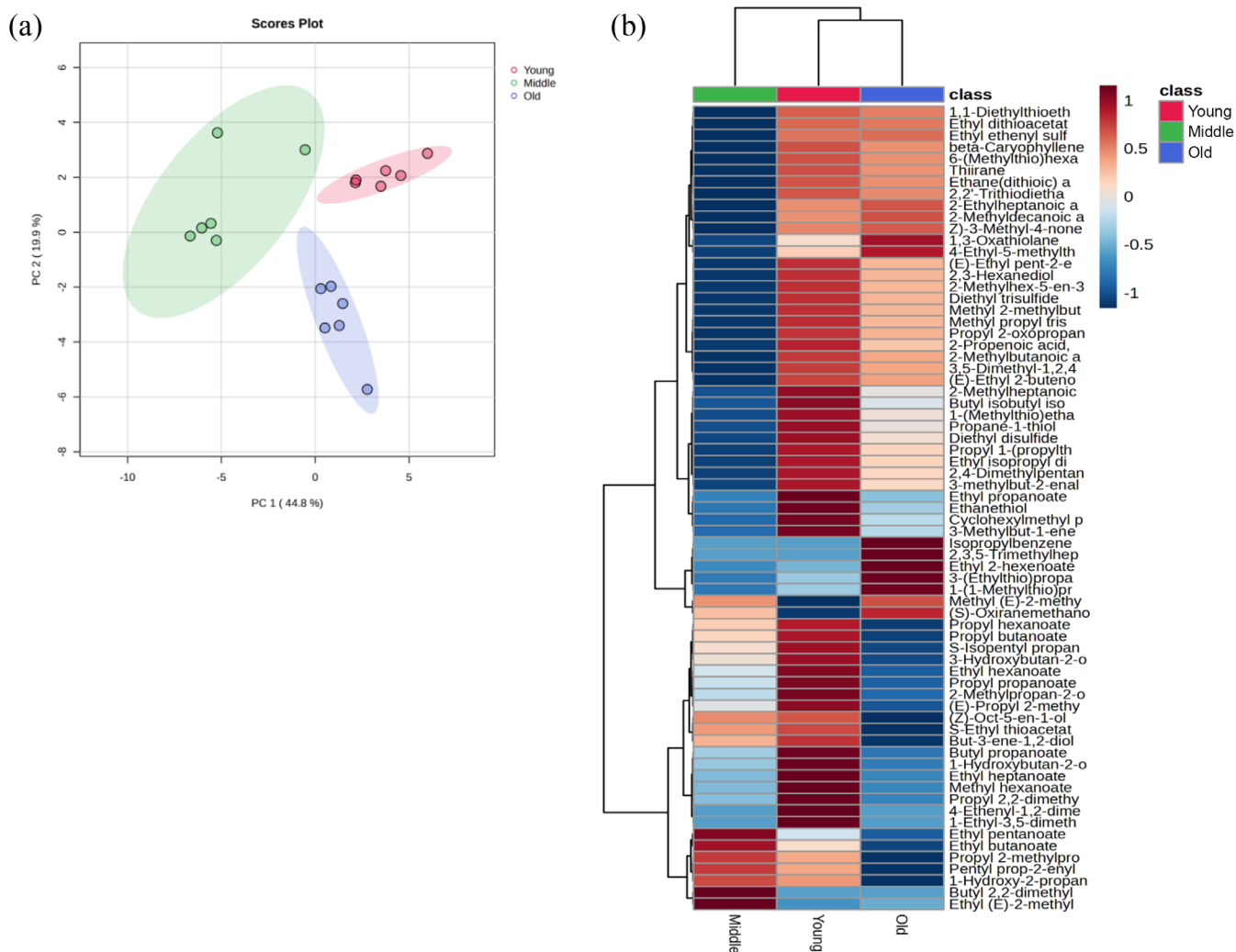


Figure 6. (a) PCA scatter plot showing the clustering of volatile metabolites in MKD from three tree age groups (young, middle, and old), with the first two components explaining 64.7% of the variance. (b) Heatmaps illustrating volatile metabolite concentrations, where brown indicates high and blue indicates low concentrations.

changes, and additionally, the current study did not examine precursor amino acids, enzyme activities, or gene expression associated with sulfur metabolism; the mechanistic basis underlying these differences needs further investigation. Similarly, propyl propanoate, a compound reported to exhibit a fruity aroma,³⁷ was higher in durian from young trees. In fruits, the volatile ester is biosynthesized through the action of alcohol acyltransferases, which catalyze the reaction between alcohols and acyl-CoA derivatives during ripening.¹⁹ However, the exact biosynthesis of esters in durian fruit has not been reported in the literature. These variations in ester compounds among tree ages may arise from differences in enzymes or precursors available, which are associated with ester biosynthesis. Plant nutrition also contributes to ester variation, as previous studies observed that nitrogen availability can influence the biosynthesis of ester in some fruit species,⁵⁵ and our earlier work on the same orchard demonstrated higher nitrogen levels in fruits from young trees.⁴² From these observations, there is a possibility that differences in nitrogen, together with physiological tree ageing, may contribute to the observed differences in ester metabolism.

Additionally, methyl (E)-2-methyl-2-butenate and 3-(ethylthio) propanoic acid were observed to be more abundant in fruit from

old trees compared to young and middle-aged trees. Chemically, these compounds are likely contributors to fruity and sulfurous aroma in fruits, respectively. However, their presence and specific roles in fruit aroma, including durian, have not been reported in the literature. Among other volatile metabolites, 1-hydroxy-2-butanone was also observed to be higher in fruits from young trees compared to middle-aged and old-aged trees. A structurally similar compound, 4-hydroxy-2-butanone, was previously reported in Malay and Thai durian to contribute to durian's sweet and caramel flavor.^{14,38} Although ketones are commonly synthesized from fatty acid metabolism,¹⁷ the biosynthetic origin of 1-hydroxy-2-butanone in durian remains unknown. Therefore, it is unclear whether its higher abundance in fruits from young trees reflects age-dependent differences in lipid metabolism, precursor availability, or enzyme activity involved in ketone biosynthesis. Further transcriptomic study is required to explain the mechanisms by which tree age regulates the formation of these volatile compounds in MKD.

The differences in percentage of fatty acids between tree ages, especially the higher content of methyl palmitoleate, hexadecanoic acid methyl ester, butyric acid methyl ester, tetradecanoic acid, 10,13-dimethyl-, methyl ester, methyl tetradecanoate, and

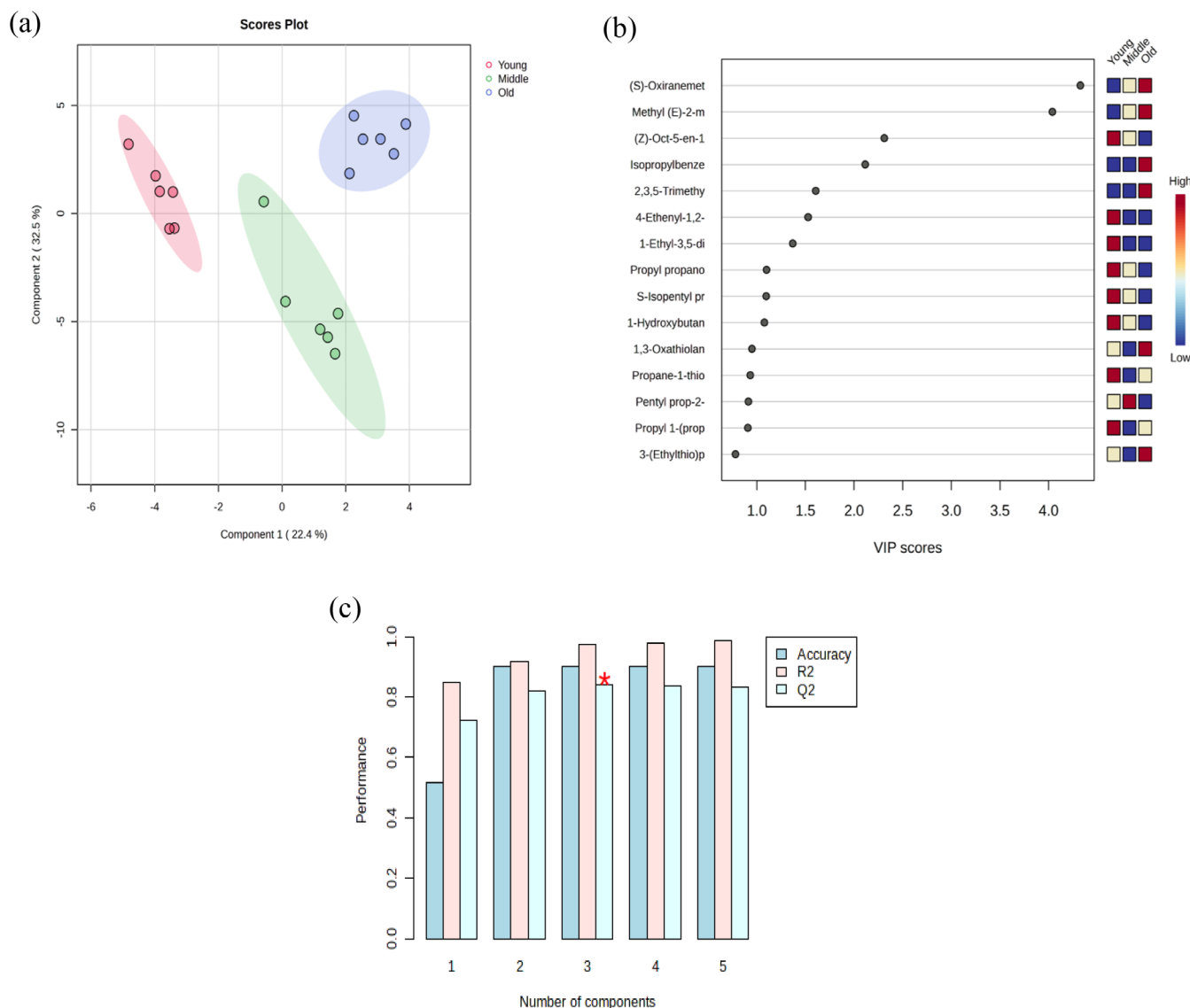


Figure 7. (a) PLS-DA of volatile metabolites in MKD from young-, middle-, and old-aged trees ($n = 6$). The PLS-DA plots display the variance in the predictive components. Component 1 (22.4%) and component 2 (32.5%) display distinct clusters among durians of different tree ages. (b) VIP displays important markers prevalent to distinguish durian from different tree ages. (c) Cross-validation parameter plots displaying R^2 and Q^2 of the PLS-DA model of MKD across different tree ages.

elaidic acid methyl ester, may be due to a high photosynthetic rate in young and middle-age trees compared with old trees. In many forest trees, high photosynthetic rates are associated with young trees.⁵⁶ High photosynthetic rates influence lipid metabolism, which increases fatty acid biosynthesis.⁵⁷ For instance, light microclimate affects the lipid profile and transcripts associated with it in photosynthetically active strawberry seeds, suggesting that photosynthetic rates fuel lipid pathways in strawberry seeds. However, we cannot rule out the possibility that the differences in fatty acid levels observed are a result of differences in photosynthetic activities, since photosynthetic activity was not measured in our study. A study by Kirca⁵⁸ observed a decrease in fatty acid composition, especially oleic acid, in hazelnuts with increasing tree age. However, the author points out these differences in terms of ecological, geographical, and soil characteristics rather than tree age. In the present study, all fruits were obtained from the same orchards under the same environmental conditions;

therefore, it is likely these differences are a result of tree ageing, although other orchard factors such as fertilization history or tree management cannot be completely overlooked.

From the integrative analysis of markers, it was revealed that the total relative concentration of sulfur compounds, esters, terpenes, alcohols, and aldehydes was higher in fruits from young trees. In contrast, fruits from middle-aged and old trees have higher concentrations of total sugars, fatty acids, organic acids, and amino acids, indicating potential differences in taste-related metabolites (Fig. 8). Since total sugars and organic acids contribute to the desirable flavor in fruits,^{12,59,60} these differences in metabolites may influence the flavor profile of MKD. However, with the absence of sensory analysis data, we cannot rule out whether these metabolite differences translate into different flavor quality of MKD fruit. Besides, these findings indicate that tree age not only influences isolated metabolites but also reprograms the primary metabolic networks that underpin MKD fruit flavor.

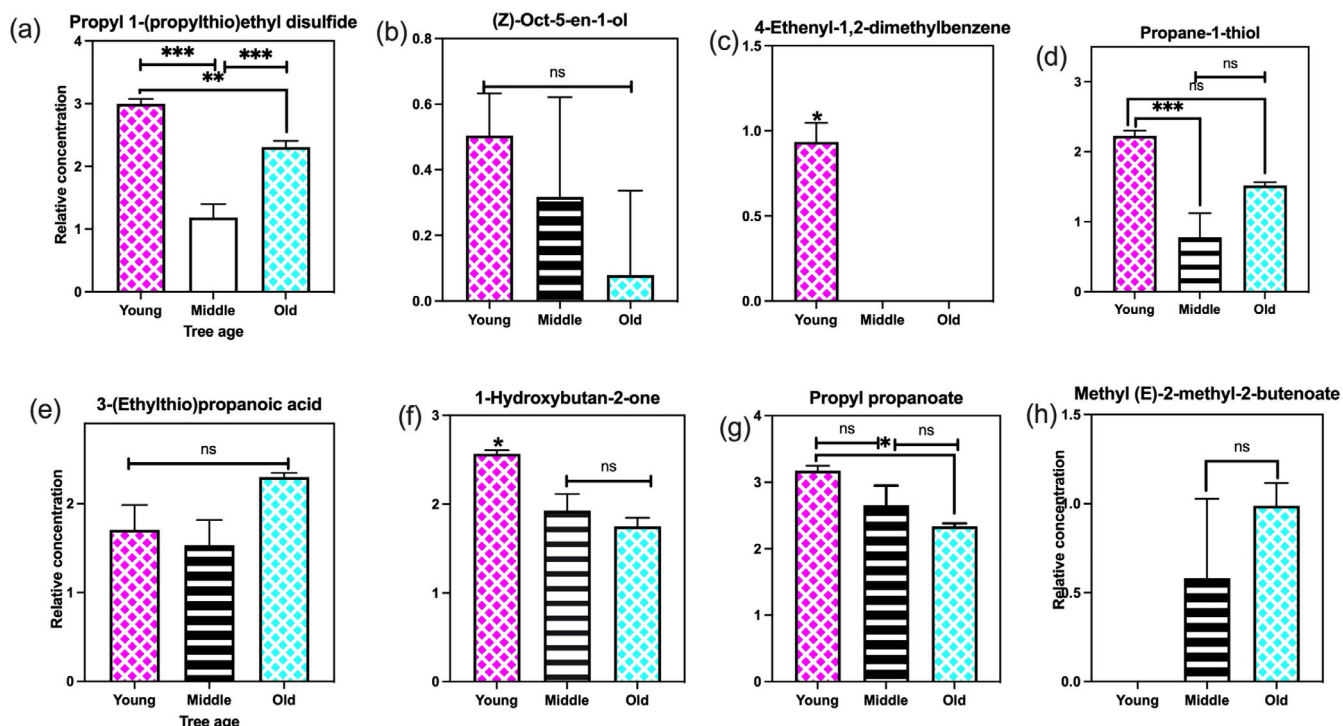


Figure 8. Changes in influential volatile metabolite markers in MKD as affected by tree age (young, middle, and old). Symbol (*) means significant differences between groups with Tukey's HSD test; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Y-axis, relative concentration; X-axis, tree age.

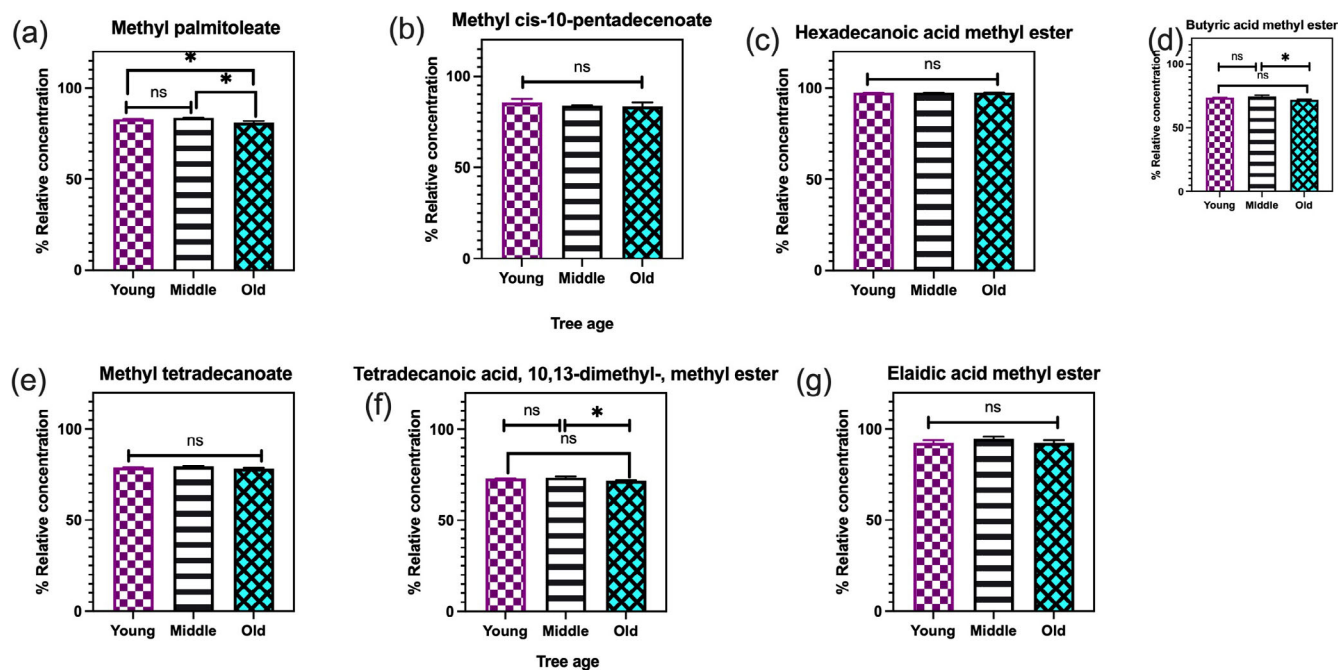


Figure 9. Means ($n = 6$) of percentage relative fatty acid composition of MKD fruits harvested from different tree ages (young, middle, and old). Symbol (*) indicates significant differences between groups based on Tukey's HSD test ($P < 0.05$), while 'ns' denotes no significant differences. Error bars represent standard error.

It is important to consider the limitations of the current study when interpreting these findings. Orchard management, especially mineral applications, influences primary and secondary metabolism. Variations in mineral supply can influence the accumulation of metabolites such as sugars and organic acids,^{61,62}

thereby contributing to differences in fruit biochemical composition. Since mineral uptake efficiency by a plant can vary with tree age,^{32,63} the absence of information on fertilization regimes and the mineral management practices of this orchard limits the interpretation of whether the observed biochemical differences are

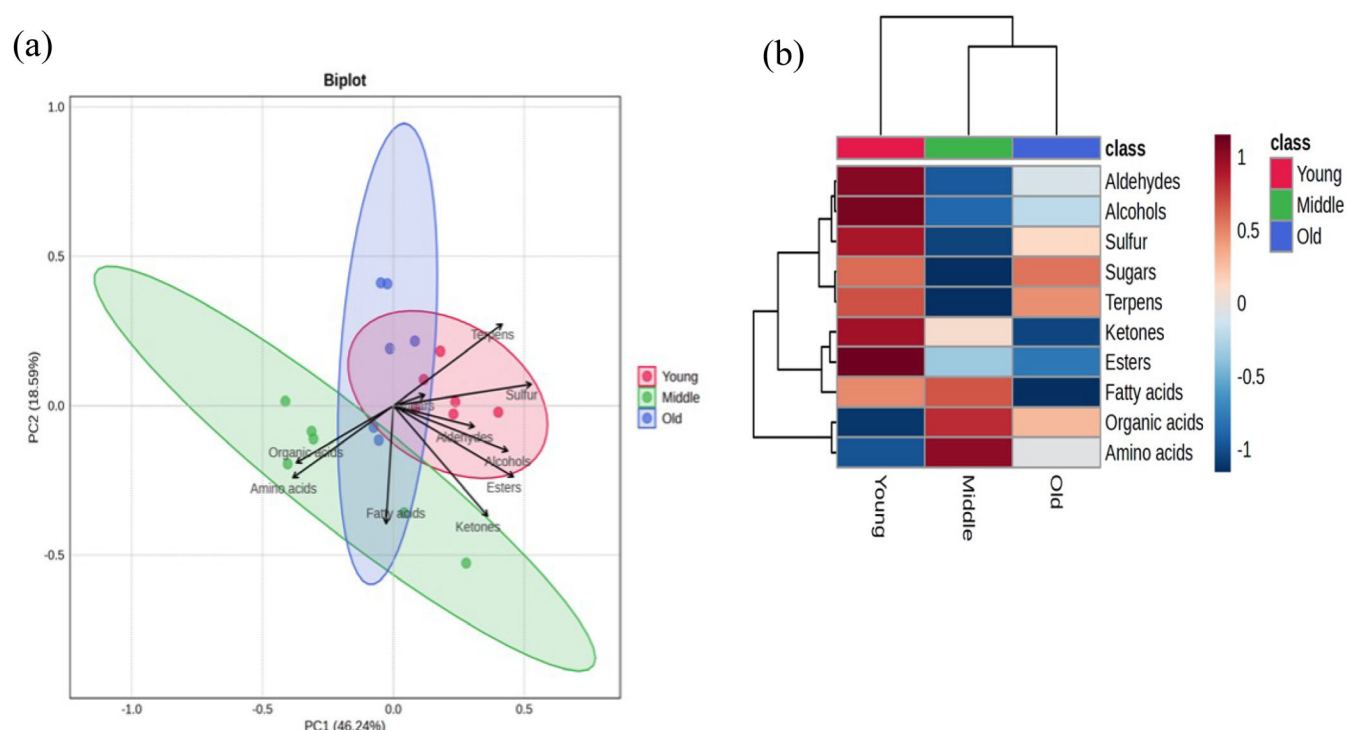


Figure 10. (a) PCA biplot reveals the total relative concentration of metabolites. (b) Heatmaps showing the concentration of metabolites in different tree ages (young, middle, and old). Heatmaps illustrating relative metabolite concentrations, where brown indicates high, and blue indicates low concentrations.

solely due to age-related changes or influenced by differences in mineral management. Furthermore, MKD fruits used in this study were collected from a single orchard, which restricts the generalizability of the findings across different growing regions, environmental conditions, and orchard management practices. In addition, although six biological replicates ($n = 6$) are a common sample size used in untargeted metabolomics studies, a larger number of replicates would increase confidence in detecting subtle metabolic differences among tree age groups. Therefore, future studies should include fertilization treatments, larger sample sizes, and fruits collected from different growing regions to better understand the impact of tree age on the metabolite composition of MKD. Furthermore, integrating metabolomics with transcriptomics and sensory evaluation would provide a more comprehensive understanding of the molecular mechanisms underlying age-related metabolic changes and their implications for MKD fruit flavor.

CONCLUSION

The impact of tree age on metabolite composition was investigated in this study. The findings revealed that tree age significantly impacted the metabolite composition of MKD fruits. Fruits from the old trees exhibited higher total sugars compared to middle-aged and young trees. In contrast, durians from middle-aged trees showed the highest levels of organic acids, amino acids, and fatty acids. Young durian trees produced fruits with high levels of volatile compounds. These findings highlight that tree age is a determinant of fruit quality, influencing flavor-related metabolites of the MKD fruit. Furthermore, this study demonstrates the potential of metabolite fingerprinting for quality differentiation and authentication of MKD fruits.

However, it is important to note that this study did not include the aroma analysis or sensory evaluation; therefore, the relationship between the metabolite abundance and flavor perception of MKD fruits cannot be established. Therefore, future studies integrating sensory evaluation with instrumental aroma analysis will strengthen our understanding of the relationship between metabolite abundance and the perceived flavor of MKD fruits from trees of different ages.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

CONFLICT OF INTEREST

The authors do not have any competing interests, direct or indirect, associated with this paper.

AUTHOR CONTRIBUTIONS

Eliwanzita Sospeter: Conceptualization; writing original draft; methodology; software; formal analysis; writing – review and editing. **Phebe Ding:** Conceptualization; writing – review and editing; supervision; data curation.

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