Metabolomic profiles and differential metabolites of volatile components in *Citrus aurantium* Changshan-huyou pericarp during different growth and development stages

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ABSTRACT

Citrus fruits possess a distinctive aroma and flavor, with *Citrus aurantium* Changshan-huyou (CACH) standing out due to their considerable edible and medicinal value. However, the volatile components (VOCs) in the CACH pericarp (CP) remain underexplored. In this study, gas chromatography–mass spectrometry (GC–MS) was utilized to qualitatively analyze VOCs in 27 CP samples across different growth stages. A total of 544 VOCs were identified, including 91 terpenoids. The types, quantities and distributions of VOCs were conducted. Detailed discussions on the major terpenoids in CP were also presented. A metabolomics approach combining multivariate statistical analysis with univariate analysis was employed for screening the differential metabolites. The study provides comprehensive insights into the VOCs in CP and citrus plants. Moreover, it delivers the first in-depth analysis of differential metabolites in CP throughout the entire CACH growth and development process, laying a foundation for ongoing research and development of the VOCs in CP.

1. Introduction

Citrus fruits are loved for their distinctive aroma and flavor, as well as their richness in nutrients and important health benefits. However, citrus plants exhibit substantial discrepancies in bioactive ingredients among different varieties, showcasing diverse germplasm resources (Lu et al., 2023). China holds the position of the world's largest citrus producer, ranking as a global leader in both citrus cultivation and production (Food and Agriculture Organization of the United Nations, 2022). *Citrus aurantium* Changshan-huyou (CACH), an endemic and valuable citrus variety in China, is a hybrid variety of *Citrus maxima* (Burm.) Merr. and *Citrus sinensis* Linnaeus, and is mainly found in counties such as Changshan, Longyou and Jiangshan in Zhejiang province. Recognized as a national geographical indication product of China, CACH has a cultivation and breeding history spanning over a century. It blooms from late April to early May, maturing in November, boasting high yield, strong adaptability, storage resistance and resistance to poverty (Jiang et al., 2022). Furthermore, the high annual production and low price of CACH offer significant potential for research and

development as a specific medicinal and edible homologous plant that is unique to Zhejiang Province (Fig. S1).

CACH is a kind of green food that offers a range of benefits, including nutritional, therapeutic, aesthetic and healthcare properties. In terms of human health and well-being, the consumption of more medicinal foods, such as CACH, has significant and far-reaching implications for people's health and well-being (Chen, 2023). The fermented juice of CACH has been proven to contain a considerable number of bioactive peptides that can improve the intestinal flora imbalance caused by obesity and also has the potential to be developed into functional healthcare products (Yan et al., 2021). The flesh and pericarp of CACH are rich in nutrients (sugars, organic acids, vitamins, minerals, dietary fiber, pectin and pigments) and bioactive compounds (coumarins, flavonoids, terpenoids). These compounds enable CACH to participate in human metabolism and regulate physiological activities, providing substantial benefits for human health (Jiang et al., 2022; Singh et al., 2020). The aroma and flavor of citrus fruits are generally imparted by VOCs. Research indicates that the main components of CACH pericarp (CP) essential oil are monoterpenoids, sesquiterpenoids, and their oxygen-

containing derivatives. ᴅ-limonene, α-pinene, myrcene, ocimene and linalool are considered the main bioactive substances (Guo et al., 2019). Numerous studies have confirmed the VOCs in CP have significant antibacterial, antioxidant, analgesic, anti-anxiety, anti-inflammatory and neuroprotective effects, which can be used as a source of functional ingredients and natural preservatives (Guo, Gao, et al., 2018). Furthermore, due to the aroma, taste and bioactivity of VOCs in CP, they are used not only as natural flavor additives in the manufacture of perfumes, flavors and other products for the food industry, but also as flavoring agents in ice cream, beverages and other food products to enhance the taste and mouthfeel of products (Singh et al., 2021).

The volatile bioactive components in CP vary considerably during different growth stages of CACH, influenced by factors such as fruit growth, cultivation environment and climate. In recent years, metabolomics technology has been widely employed to unveil changes in

various endogenous metabolites during citrus fruit development. It serves as a practical tool for monitoring and comparing changes in chemical profiles throughout the development of research subjects. Miao et al., 2023 comprehensively characterized the constituents of Aurantii Fructus using multiple liquid chromatography–mass spectrometry (LC–MS)-based metabolomics approaches, systematizing both LC and MS characteristics of polymethoxyflavones. Based on metabolomics analyses, Kim et al., 2021 compared the total flavonoid content of different citrus varieties. Flavonoids and limonoids were identified as the primary chemical constituents of citrus peel and seeds by Zhang et al., 2022. Yan et al., 2021 utilized metabolomics to analyze representative differential metabolites during the growth and development of *Citrus wilsonii* Tanaka, concluding that naringenin might be a biomarker for it. Current studies have mainly focused on flavonoids in citrus plants, with limited attention given to essential oil constituents such as

Fig. 1. Venn diagram of CP with different ripeness levels based on GC–MS analysis. The overlapping regions of the Venn indicate the metabolites shared between corresponding groups in the experimental comparisons, and the remaining regions show the specific metabolites.

sesquiterpenoids. However, it is a fact that the high VOCs in CP are not only the main source of odor in CACH, but also have a wide range of applications in food processing, storage and quality. Therefore, conducting more in-depth and detailed studies on the VOCs in CP is crucial in employing metabolomics.

In this study, gas chromatography–mass spectrometry (GC–MS) analysis was used to comprehensively and systematically investigate the VOCs present in CP during different growth periods. Among them, terpenoids were the main constituents of CP essential oils and found extensive applications as natural flavors and fragrances in the food, beverage, cosmetic, pharmaceutical, textile, and leather industries (Jiang & Wang, 2023). Terpenoids have great contributions to human health and production. Based on this, the terpenoids in CP have been discussed in detail in this study. Simultaneously, metabolomics methods combined with multivariate statistical analysis were also employed to visualize the correlation of VOCs and to search for differential metabolites between different growth stages of CACH. This study aimed to elucidate the relationship between VOCs in CP and the harvesting period, and explore the types, distribution patterns and content changes of VOCs during the growth and development of CACH. These findings are intended to provide a theoretical basis and scientific evidence for determining the harvesting period, to promote the development and utilization of CACH resources, and to provide guidance for the development of CACH in various industries.

2. Materials and method

2.1. Plant material

The CACH samples (including roots, leaves, flowers, fruits, and seeds) were collected from Changshan County, Quzhou City, Zhejiang Province (28°55′21″N, 118°35′41″E), choosing robust CACH trees that displayed consistent growth in the orchard. The picking occurred between May and October 2023 at one-week intervals, resulting in 27 CP sample groups. Simultaneously, climatic conditions during the picking were recorded (Fig. S2, Table S1). The CACH samples were appropriately labeled and preserved in a refrigerator at −40 °C.

2.2. VOCs extraction

After freezing pretreatment, the CP samples were peeled and cut into pieces. 0.10 g of the sample was added with 1.00 mL of chromatographic n-hexane (Merck KGaA, Darmstadt, Germany) and extracted for 60 min at 500 W ultrasonic power and 50 °C before filtration (0.22 μm pore size) and then sealed in sample vial at 4 °C for subsequent GC–MS analysis. Each group of samples was replicated five times. Samples from different parts of CACH were manipulated as described above.

2.3. Qualitative analysis of VOCs based on GC–MS

VOCs analysis of CP was performed on a GCMS-QP2010 ULTRA (Shimadzu, Kyoto, Japan). A DB-5 ms fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J&W scientific, Folsom, CA, USA) was utilized to separate the samples. High-purity helium (purity not <99.999%) served as the carrier gas with a constant flow rate of 1.0 mL/min. The injection port temperature was 250 °C with no split flow injection and a 5-min solvent delay. Temperature programming: 40 °C for 2 min, 5 °C/min to 150 °C, and hold for 2 min, 4 °C/min to 250 °C, and hold for 3 min. The ion source and transmission line temperatures were 230 °C and 280 °C, respectively, and the electron energy was 70 eV. Scanning occurred in full scan mode (scan) with a quality scanning range was m/z: 50–550. Identification of VOCs was accomplished by comparing their mass spectra and retention time with those stored in the NIST17 database.

2.4. Statistical analysis

The experimental data were organized using Microsoft Excel 2019 (Microsoft Corp., Redmond, USA). The structural formulae of the VOCs were drawn using Chem Draw 20.0 (PerkinElmer Corp., Waltham, USA). The systematic clustering heat map was generated using TBtools-II (https://github.com/CJ-Chen/TBtools/releases) (Chen et al., 2023). Principal component analysis (PCA), partial least square multiplicative discriminant analysis (PLS-DA), and score plots were conducted using SIMCA 14.1 (Umetrics, Umea, Sweden). Origin 2021 (OriginLab Corp., Hampton, USA) was employed to plot score plots, Venn plots, and others.

3. Results and discussion

3.1. Identification of VOCs by GC–MS

The VOCs in each sample were analyzed by GC–MS, resulting in the identification of 544 VOCs (Fig. S3, Table S2). These compounds were categorized into 12 distinct chemical classes based on their chemical structures: terpenoids, alkanes, alkenes, alicyclic hydrocarbons, aromatic compounds, alcohols, ethers, aldehydes, ketones, fatty acids, esters and others. Terpenoids, being crucial components in the VOCs in CP and are key determinants of its distinctiveness compared to other species within the same genus, are further classified into monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids, with the oxygenated derivatives considered as part of the terpenoid category.

3.1.1. Differential analysis of VOCs at different stages

The aroma and flavor of citrus fruits are associated with changes in VOCs. Venn diagrams were employed to elucidate the unique or common VOCs in CP at different stages of growth and development, providing a reference for exploring changes in aroma and flavor. The growth and development of CACH can be roughly divided into 3 different stages, including cell division, cell enlargement and fruit maturity (Lu et al., 2017). Fig. 1A-C illustrated differences in VOCs during the initial stage of CACH growth and development-the cell division stage (CACH01-CACH07), characterized by CACH transitioning from the fruit set stage, evolving from an ovary into a young fruit with slow growth and development. Only 52 metabolites were shared between sample sets at this stage, and the composition differences were notable. CACH04, in particular, exhibited significantly more characteristic metabolites than other sets during this stage. Fig. 1D-E depicted differences in the VOCs of CACH during the subsequent stage-the cell enlargement stage (CACH08-CACH15), marked by accelerated fruit expansion. By this point, key VOCs in CP had emerged, and the VOCs in each group appeared to have stabilized, resulting in a significant reduction in differences between sample groups compared to the initial stage. Research has indicated a decreasing trend in the main phytochemical properties as fruit parts mature throughout development (Feng et al., 2021). The research results indicated a decline in the total number of compounds detected in CP as CACH entered the third stage of fruit maturity stage. Additionally, the number of specific metabolites displayed by each group stabilized in a lower range, consistent with the aforementioned study (Fig. 1F-H). In addition to discussing the differences in VOCs between CPs, this study further investigated the differences in VOCs in different parts of the CACH, providing a basis for subsequent related studies. Highly significant differences were observed in the VOCs present in CACH27 and the roots, with the roots exhibiting the highest number of volatile constituents and the seeds displaying the lowest number (Fig. 1I). In summary, the VOCs present in CP showed notable dissimilarities during the initial growth and development stages. However, as CACH matured and entered the cell enlargement stage, the divergence in VOCs decreased and eventually reached a steady state, and the aroma and taste of CACH gradually developed with maturity.
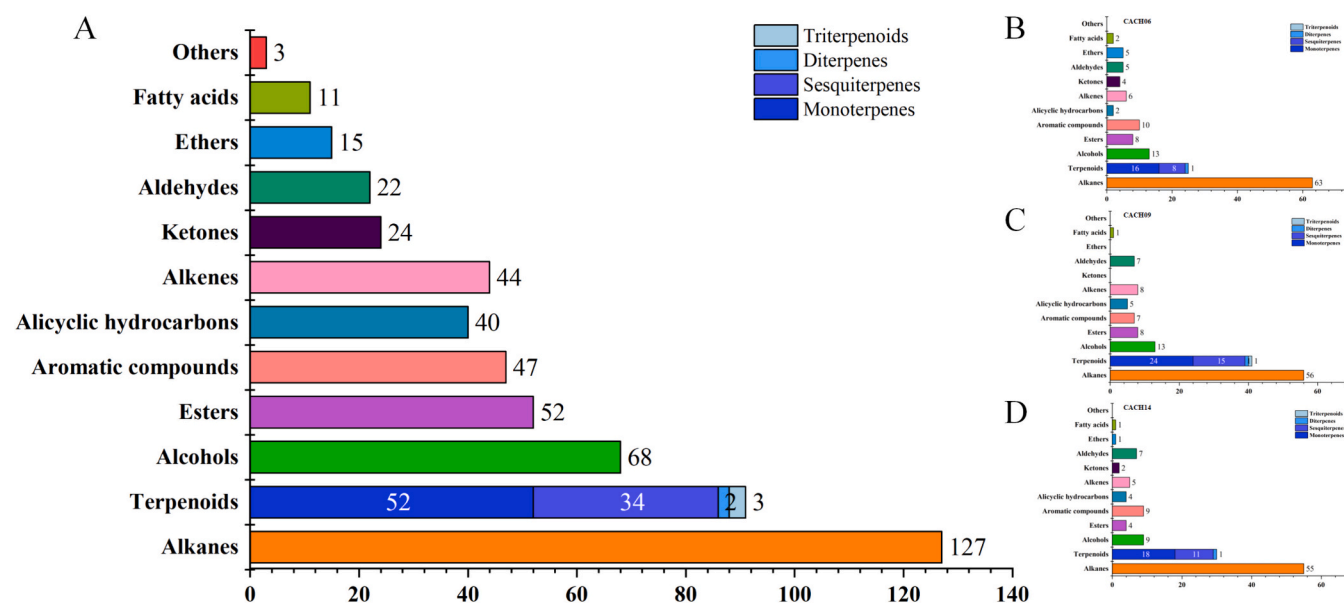
Fig. 2. Category and quantity of VOCs in all samples (A) and in CACH06, CACH09 and CACH14 (B–D).

3.1.2. Differential analysis of various categories of VOCs

The number of VOCs in each class was as follows: 127 alkanes, 44 alkenes, 91 terpenoids (52 monoterpenoids, 34 sesquiterpenoids, 2 diterpenoids, 3 triterpenoids), 40 alicyclic hydrocarbons, 47 aromatic compounds, 68 alcohols, 15 ethers, 22 aldehydes, 24 ketones, 11 fatty acids, 52 esters, 3 others (Fig. 2A). Of these, 14 alkanes, 14 terpenoids (9 monoterpenoids and 5 sesquiterpenoids) and 1 alcohol were detected at all stages of CACH development, such as tetradecane (CA244), γ-terpinene (CA099), cis-β-copaene (CA250) and 2-hexyldecanol (CA215) (Table 1). This study surpassed similar reports by detecting diterpenoids and triterpenoids in CP for the first time, along with uncovering 122 alkanes, 71 terpenoids, 64 alcohols, 50 esters, 44 aromatic compounds, 40 alicyclic hydrocarbons, 44 olefins, 23 ketones, 17 aldehydes, 15 ethers and 10 fatty acids in CP (Gao et al., 2022).

Alkanes were the most diverse of the 12 classes of VOCs in this study, followed by terpenoids and alcohols. Alkanes were associated with the cuticle layer on the surface of CP. The cuticular wax composition of citrus mainly comprised ultra-long chain aliphatic hydrocarbons and their oxygenated derivatives, including alkanes, aldehydes, alcohols, fatty acids, esters, ketones and cyclic compounds. As CACH developed and matured, wax-like substances accumulated on the surface pores of the fruit skin to reduce fruit water loss and have a preservation effect. The chemical composition of cuticular waxes exhibited wide variation during fruit development due to the combined effects of a variety of environmental factors such as wind, temperature, light, pre-harvest treatment and bagging (Zhou et al., 2022). Probably due to the above reasons, alkanes in CP varied greatly during growth and development (Fig. S4), with CP containing more alkane species during the cell division and enlargement stages, such as 51 alkanes in CACH03, 63 alkanes in CACH06, 64 alkanes in CACH08 and CACH15. As CACH entered the maturity stage, alkane species gradually stabilized, suggesting that the changes in alkane species were more influenced by the environment in the early stage of CACH growth and development, consistent with the above report (Zhou et al., 2022).

Terpenoids, the most characteristic of the VOCs in CP and variations in their contents and species mainly affect the flavors of CP, are often considered characteristic compounds of the VOCs in CP. Unlike the alkanes, no clear trend in terpenoid species changes throughout the 3 stages of CACH development. The proportion of monoterpenoids in each group showed a decreasing trend as CACH matured, while the sesquiterpenoid levels increased. Analysis in synthetic biology suggests that

specific enzymes may convert some monoterpenoids into sesquiterpenoids during the maturation process of CACH. Accumulation of precursors, including amino acids, glycosides, and fatty acids in the pregrowth period of CACH, is likely responsible for the observed terpenoid alterations. This accumulation offers sufficient substrates for relevant synthesis, enhancing the synthesis of certain terpenoids (Zhang et al., 2019). This study discovered that diterpenoids and triterpenoids were mainly present in the second stage. Furthermore, triterpenoids were only detected in CACH08, CACH12, CACH14 and CACH25. Based on the characteristics of terpenoids in the different sample groups, CACH06, CACH14 and CACH24 were selected as representative sample groups among the three growth phases (Fig. 2B-D). CACH06 had a lower number of terpenoids (25) and sesquiterpenoids (8) than other samples. Moreover, CACH14 had the highest number of both monoterpenoids (24) and sesquiterpenoids (15), as well as the highest total number of triterpenoids (41), while CACH24 had the least number of monoterpenoids (15). Combined with the changes in terpenoid species, it was hypothesized that the terpenoids present in the second stage of CACH could serve as characteristic compounds, which are deemed the foundation for evaluating the flavors constituents of CP.

Alcohols, esters, aldehydes and ketones are not only components of the cuticular wax of CP, but together with terpenoids influence the flavor and aroma of CACH. Those and other compounds present in CP undergo complex changes during development, influenced by factors such as climate (especially low temperatures), enzyme activity, metabolism, and degradation (Liu et al., 2022). Such changes may result in species variability that cannot be elaborated extensively in this discussion.

3.2. Mechanism and functions of terpenoids in CP

Terpenes and terpenoids constitute the main bioactive constituents present in plant essential oils. Their antimicrobial activity against foodborne microorganisms and food preservative properties would make them an excellent alternative to the standard bactericides and fungicides currently used in the food industry (Hao & Liu, 2023; Masyita et al., 2022). In addition, terpenoids have strong pollinator-attractive characteristics that protect plants from invasion by foreign substances, including microorganisms and insects (Li et al., 2020). Terpenes are naturally produced by plants and are exceedingly diverse, biosynthesized by two pathways - the mevalonate pathway and the 2-C-

Table 1
Category and quantity of VOCs in CACH01–CAHC27.

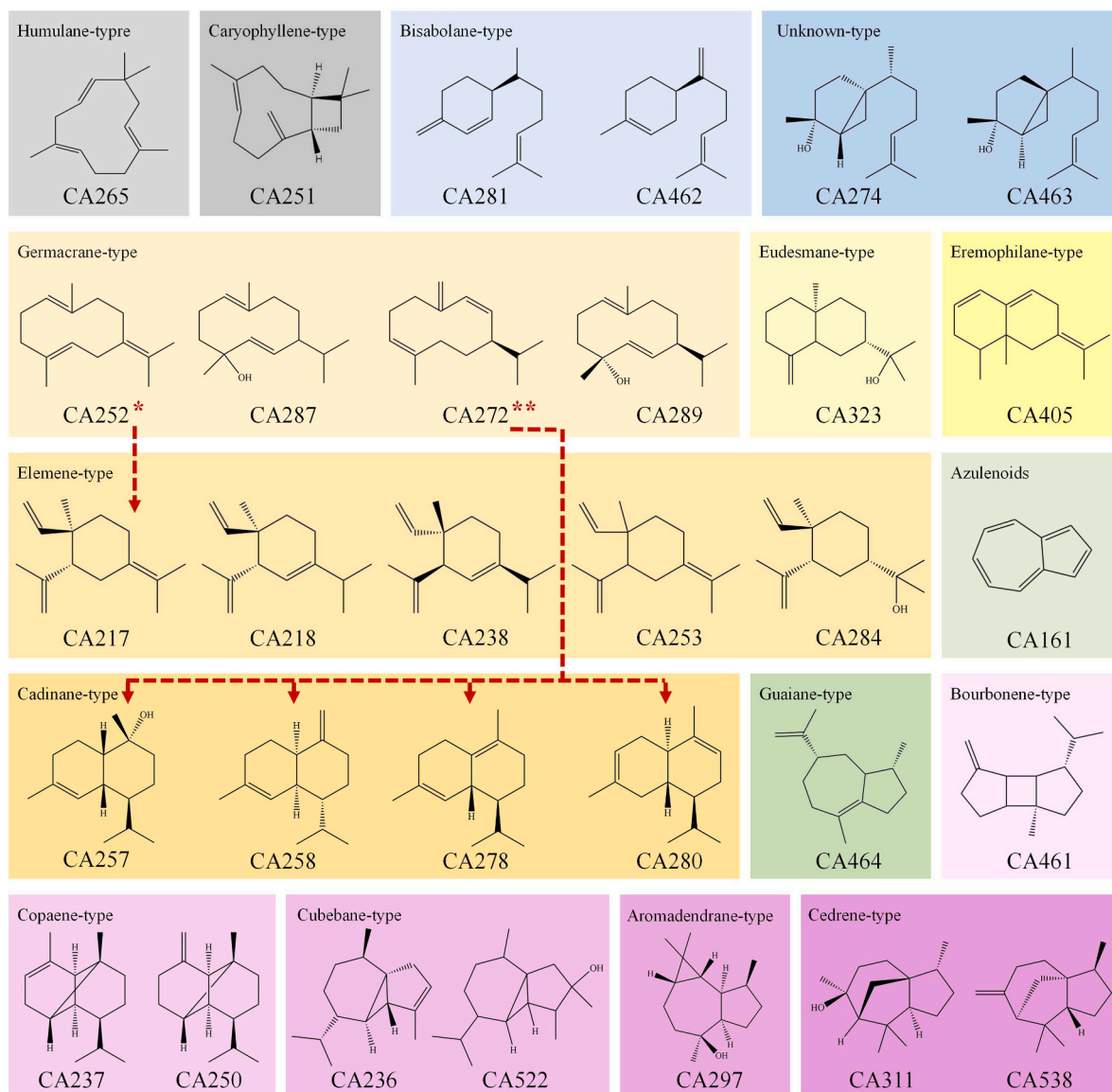| Category	Alkanes	Terpenoids	Monoterpenoids	Sesquiterpenoids	Diterpenoids	Triterpenoids	Alcohols	Esters	Aromatic compounds	Alicyclic hydrocarbons	Alkenes	Ketones	Aldehydes	Ethers	Fatty acids	Others
CACH01–27	127	91	52	34	2	3	68	52	47	40	44	24	22	15	11	3
CACH01	54	33	19	13	1	0	14	8	12	8	4	2	5	1	3	0
CACH02	60	38	23	14	1	0	13	9	15	4	10	1	5	2	1	0
CACH03	51	31	21	10	0	0	12	6	12	4	9	2	6	4	2	0
CACH04	54	35	23	12	0	0	15	8	14	10	4	3	5	2	1	0
CACH05	59	37	22	14	1	0	13	8	8	8	7	2	4	2	1	0
CACH06	63	25	16	8	1	0	13	8	10	2	6	4	5	5	2	0
CACH07	59	35	23	12	0	0	17	8	11	7	6	4	5	3	2	0
CACH08	64	37	21	14	1	1	14	8	12	9	6	2	7	1	2	0
CACH09	54	32	19	12	1	0	15	10	10	11	7	0	6	4	2	0
CACH10	59	33	21	11	1	0	10	10	10	5	7	2	7	4	1	0
CACH11	56	34	21	12	1	0	13	14	8	7	8	4	5	3	2	1
CACH12	55	34	20	11	1	1	22	7	8	7	7	0	6	2	3	1
CACH13	57	36	20	15	1	0	12	7	10	4	8	1	7	1	2	1
CACH14	56	41	24	15	1	1	13	8	7	5	8	0	7	0	1	0
CACH15	64	35	20	14	1	0	12	9	7	8	7	1	7	2	2	0
CACH16	59	34	19	15	0	0	9	7	8	2	5	0	5	2	3	0
CACH17	55	32	18	13	1	0	16	7	10	1	6	1	4	2	3	0
CACH18	49	34	22	11	1	0	11	6	8	2	9	2	8	3	2	0
CACH19	55	30	18	11	1	0	9	4	9	4	5	2	7	1	1	0
CACH20	55	27	16	11	0	0	13	4	7	6	7	1	5	2	2	0
CACH21	57	29	18	11	0	0	8	4	6	3	6	1	5	2	2	0
CACH22	52	30	19	11	0	0	10	9	7	1	6	1	6	3	1	0
CACH23	59	30	18	11	1	0	11	5	9	2	7	1	5	3	2	0
CACH24	52	29	15	13	1	0	9	3	8	4	3	1	4	2	2	0
CACH25	59	31	17	13	0	1	12	6	8	0	4	2	5	1	1	0
CACH26	47	36	20	15	1	0	11	5	6	0	7	0	8	2	1	0
CACH27	49	30	18	12	0	0	12	4	5	1	5	2	0	1	2	0

Fig. 3. Synthesis pathways of monocyclic monoterpenoids (A) and bicyclic monoterpenoids (B) in CP based on structural analysis. (* confirmed synthesis pathway, and the six compounds in the black box are natural menthane-type monoterpenes in CP). Structural formula diagram of sesquiterpenoid compounds (C).

methylerythritol 4-phosphate pathway. All terpenoids are formed through the sequential condensation of isopentenyl diphosphate and dimethylallyl diphosphate (Richter et al., 2015). The mechanisms of terpenoid formation during the growth or storage of CACH and their potential as functional food ingredients were analyzed and discussed in depth in the study.

C

Humulane-type: CA265

Caryophyllene-type: CA251

Bisabolane-type: CA281, CA462

Unknown-type: CA274, CA463

Germacrane-type: CA252 *, CA287, CA272 **, CA289

Eudesmane-type: CA323

Eremophilane-type: CA405

Elemene-type: CA217, CA218, CA238, CA253, CA284

Azulenoids: CA161

Cadinane-type: CA257, CA258, CA278, CA280

Guaiane-type: CA464

Bourbonene-type: CA461

Copaene-type: CA237, CA250

Cubebane-type: CA236, CA522

Aromadendrane-type: CA297

Cedrene-type: CA311, CA538

* CA252 synthesized into CA217
** CA272 synthesized into CA257, CA258, CA278 and CA280

Fig. 3. (continued).

3.2.1. Monoterpenoids

Monoterpenoids are a group of compounds with a 10-carbon skeleton, polymerised with two isoprenoids in the backbone. The majority of monoterpenoids possess potent aromas and exhibit a range of biological activities beneficial to human health, including anticancer, antibacterial, antioxidant and antiviral effects. These compounds have received widespread application in food, cosmetic, agricultural and pharmaceutical industries (Zielińska-Błajet & Feder-Kubis, 2020). Based on the number of carbon rings contained, monoterpenoids were classified into structural types including acyclic (21), monocyclic (18) and bicyclic monoterpenoids (13), and no tricyclic monoterpenoids were detected in this study (Fig. 3), which is in agreement with the reports of already available studies (Zhang et al., 2023). The study classified the VOCs detected from a biosynthetic perspective and the oxygenated derivatives of monoterpenes were classified as monoterpenoids, including monoterpene alcohols, ketones, and esters. Monoterpene esters are formed by the esterification of monoterpene alcohols with short linear acyl groups mediated by coenzyme A (CoA). These monoterpene esters are commonly used as flavor adjuvants in the food, pharmaceutical, and cosmetic industries. They have been recognized as safe for human consumption by the US Food and Drug Administration (FDA) and the European Union (Staudt et al., 2022). In this study, 38 monoterpenoids were detected in CP for the first time, including (*E*)-*β*-terpinolene (CA115), (−)-*α*-terpineol (CA167), *β*-terpineol (CA275), *α*-ocimene (CA458) and (−)-*β*-citronellene (CA529), et al.

Acyclic monoterpenoids have a smaller number of structural types, including mycrane-type, lavandulane-type and artemisane-type, and they are often involved in secondary transformations and couplings that lead to the creation of biologically active end products. In addition, their uncyclized subsets are more reactive than their cyclized counterparts, as evidenced by their greater ease of conversion to compounds such as monoterpene aldehydes, acids, and esters (Bergman et al., 2023). In this study, 17 mycrane-type monoterpenoids and 4 artemisane-type monoterpenoids were detected in CP, and no lavandulane-type monoterpenoids were detected (Fig. 3A). The study provides a clearer and more detailed understanding and investigation of the content and

distribution of monoterpenoids in the volatile constituents of CP compared to similar studies (Guo, Gao, et al., 2018). β-myrcene (CA073) and citronellal (CA145) were detected throughout the development of CACH. As CACH matured, the relative content of CA073 increased steadily, and it can induce apoptosis via caspase induction. Therefore, consuming more CACH or CP-processed food can prevent and control the risk factors of human carcinogenicity from the aspect of food and diet (Bai & Tang, 2020; Wen et al., 2022). Furthermore, CA073 can be employed as a natural food preservative in a variety of products, including dried fruits, green gram seeds, orange fruit juice, and others, due to its notable antimicrobial properties (Masyita et al., 2022). CA145 has a distinctive spicy citrus and floral aroma, and is an industrial precursor to menthol, the primary ingredient in synthetic peppermint oils. Menthol modulates the flavor of food, freshens breath, and is frequently used in oral hygiene products (Bergman et al., 2023). Linalool (CA118) was primarily detected during the pre-developmental phase of CACH, with its relative content progressively decreasing as CACH matured. The changes in the relative amounts of the above compounds may be related to the metabolic activity and synthesis of the corresponding enzymes. Nevertheless, the biosynthesis of acyclic monoterpenoids has been comparatively understudied compared to that of cyclic monoterpenoids and, therefore, is not elaborated upon in detail (Kim et al., 2022).

The principal structural types of monocyclic monoterpenoids include menthane-type, cycloartane-type and stilbene-ketone-type, with menthane-type monoterpenoids prevailing in nature. In this study, all the monocyclic monoterpenoids detected in CP were of the menthane-type (Fig. 3B). Menthol which is the most characteristic compound, was not detected in CP, and iridoid compounds were not found in CP, and this result was consistent with other studies (Singh et al., 2021). The monocyclic menthane often occurs in eight conformations in plant natural products, six of which are found in CP, of which β-phellandrene (CA069), α-phellandrene (CA078), α-terpinene (CA085), D-limonene (CA090), and (E)-β-terpinolene (CA115) are generated by deprotonation quenching of the terpinyl cation. In addition, the terpinyl cation can undergo conversion by γ-terpinene synthase through the 6,7-hydride shift to terpinene-4-yl cation, ultimately yielding γ-terpinene (CA099) after proton elimination. When the α-terpinyl cation is captured by one water molecular by terpineol synthase, it eventually forms terpineol (CA159) (Lei et al., 2021). Limonene is the most prevalent menthane-type monoterpene in nature and exists in two enantiomers. The (+)-limonene enantiomer is present in the pericarp of citrus fruits and across the Rutaceae family. It is the source of citrus aroma and flavor, as well as demonstrating significant antimicrobial coordination and the ability to limit the viability of *Escherichia coli*. This provides scientific justification for its use as a preservative for milk and dairy products (Falleh et al., 2019). On the other hand, (−)-limonene is found predominantly in the Labiatae family such as peppermint and perilla (Kvittingen et al., 2021). Studies have demonstrated that limonene serves as a precursor for various other menthane-type monoterpenoids, such as limonene-1,2-epoxide ((E)-limonene oxide (CA129) and limonene oxide (CA460)), α-terpineol (C167) and carvacrol, (−)-carvone (CA187) and (+)-carvone (CA188). Consequently, it is hypothesized that CA090 could be the origin of the majority of monoterpene derivatives in CP through a series of different reactions, involving deionization, protonation and oxidation facilitated by a variety of modifying enzymes (Ren et al., 2020). Owing to the specificity of the enzymes involved in the reaction, leading to intricate and varied synthetic pathways, a detailed discussion on this matter is not provided. Regarding compound distribution and relative content, CA090, CA115, CA159 and (−)-α-terpineol (CA167) were consistently detected throughout the growth and development of CACH. Particularly, the relative content of CA090 significantly increased during the pre-growth period of CACH (CACH01-CACH08), reaching a maximum of 55.10% in CACH14. This indicates that CA090 is the primary VOC of CP, aligning with findings from other studies (Tocmo et al., 2020).

Bicyclic monoterpenoids exhibit considerable diversity, with the pinane and camphene types being the most stable, resulting in a high number of formed derivatives (Alsharif et al., 2023). The study identified 5 pinane-type, 4 thujkane-type, 1 carene-type and 1 camphene-type bicyclic monoterpenoids in CP. It is suggested that (−)-isopinocampheol (CA104), isopinocampheol (CA106) and myrtenol (CA116) may be derivatives of α-pinene (CA049). CA045, CA049 and CA070 can be detected during the entire growth and development of CACH, and CA049 has been used as a food preservative additive in dairy products such as cakes, fruit juices, minced beef, salami and milk (Falleh et al., 2019; Masyita et al., 2022). Due to their generally low relative levels in CP, bicyclic monoterpenoids are not investigated in detail.

3.2.2. Sesquiterpenoids

Sesquiterpenoids are not only the most abundant and versatile group of plant essential oils, but also a representative class of VOCs in CP, accounting for 8.2% of all detected components. In this study, 12 monocyclic sesquiterpenoids, 11 bicyclic sesquiterpenoids and 1 tricyclic sesquiterpenoid were detected in CP and no acyclic sesquiterpenoids (Fig. 3C). There is a common understanding of the origin of sesquiterpenoids in plants: all sesquiterpenoids share a common precursor, farnesyl diphosphate (FPP), which undergoes isomerization and cyclisation to give a variety of sesquiterpenoid structures. Specific aromatic properties (balsamic, spicy and woody notes) and potential health benefits (anti-inflammatory, antimicrobial and anticancer properties) of sesquiterpenoids have been increasingly reported, such as a reduction in the risk of chronic diseases (cancer and cardiovascular diseases) when consuming foods high in sesquiterpenes (Li et al., 2020). Although the volatility of sesquiterpenoids is lower than that of monoterpenoids, both have the effect of repelling herbivorous insects or attracting pollinating insects, thus reducing pathogen infections and acting as protective and reproductive agents (Beran et al., 2019).

The monocyclic sesquiterpenoids in CACH were classified into germacrane-type, elemene-type, humulene-type and bisabolane-type based on synthetic pathways and structural features. The germacrane-type sesquiterpenoids including germacrene B (CA252), germacrene D (CA272), germacrene D-4-ol (CA287) and β-germacrenol (CA289). CA252 was detected from the beginning of the 7th sample of CP until fruit ripening. It has been identified as a compound with a warm, sweet, woody-pungent, geranium-like odor and is an important flavor component of lime peel oil (Xu & Dickschat, 2023). A high relative level of CA272 was observed in CP, which exhibits antioxidant, fungicidal and insecticidal properties. These properties protect CACH from microorganisms and pests during growth and ripening. Furthermore, CA272 can be employed for food preservation and grain storage (Tourabi et al., 2023). CA287 and CA289 exhibit profound differences in distribution and content. It is hypothesized that they may be derived from distinct germacrene A synthase (GAS) catalyzing the shared precursor FPP, resulting in different germacrane-type sesquiterpenoids analogues (Jiang & Wang, 2023). It has been demonstrated that CA287 exhibits antifungal properties and can be employed as an alternative to synthetic chemical fungicides for the protection of stored grains. This alternative offers greater benefits to human health and the environment than the use of fungicides (Juárez et al., 2016). Germacrane-type sesquiterpenoids tend to undergo Cope rearrangement at elevated temperatures, resulting in the rearrangement of germacrene A, B, and C, producing corresponding β, γ and δ elemene. GAS is a key enzyme in elemene biosynthesis, which concludes with the termination of the reaction by proton departure or the intervention of water molecules, leading to the production of corresponding sesquiterpene alkenes and alcohols (Harms et al., 2020). The study identified several elemene-type sesquiterpenoids in CP, including γ-elemene (CA217), δ-elemene (CA218), (−)-β-elemene (CA238), elixene (CA253), and elemol (CA284). Moreover, CA217, CA218, CA238, and CA284 were consistently present during the growth of CACH, aligning with findings in related studies (Xu & Dickschat, 2023). CA284 is a type of elemol likely formed through the oxidative reduction of CA217 or CA253 by relevant enzymes, based on its

distribution, content and structure. The three primary conformations of elemene found in nature are β, γ and δ. Of these, β-elemene is the most active, exhibiting anticancer properties. Consumption of CACH and other food products riched in CA238 can help to prevent cancer to a certain extent (Chen et al., 2021). In addition to the previously identified monocyclic sesquiterpenoids, the humulene-type sesquiterpenoid: caryophyllene (CA265) and the bisabolane-type sesquiterpene: β-sesquiphellanderene (CA281) and β-bisabolene (CA462) were found in CP.

The types of bicyclic sesquiterpenoids detected in CP are more complex and are classified as caryophyllene-type, cadinane-type, eudesmane-type, eremophilane-type, guaiane-type sesquiterpenoids and azulene compounds. The caryophyllene-type sesquiterpenoid detected in CP essential oil was β-caryophyllene (CA251), which has been demonstrated to have protective and preventive effects on the heart, liver, nerves, kidneys, and stomach. Furthermore, it can be used as a nutraceutical and functional food (Hashiesh et al., 2021). In addition, CA251 has been shown to have a hypoglycemic effect, which may be one of the reasons why CACH is a suitable fruit for consumption by diabetic individuals. Currently, the biosynthetic pathway of caryophyllene-type sesquiterpenoids has been elucidated: utilizing FPP as a substrate, catalyzed by terpene synthase, to produce caryophyllene in different configurations. CA265 is the ring-opening isomer of CA251. Caryophyllene has a distinctive aroma, with notable antibacterial, antioxidant and insecticidal properties. It can be employed as a natural insect repellent and a preservative for foodstuffs such as bread, fruit juice and milk (Masyita et al., 2022). The cadinane-type sesquiterpenoids identified in CP were α-muurolene (CA257), γ-muurolene (CA258), γ-cadinene (CA278), and β-cadinene (CA280). The cadinane-type sesquiterpenoids are synthesized by sesquiterpene synthase using FPP as a precursor to generate the fundamental skeleton, among which CA278 was produced from germacrene D through proton transfer and conformational change (Nguyen et al., 2019). The principal active ingredients in the medicinal food Laoxianghuang, a distinctive medicinal food from Guangdong Province, China, namely CA278 and CA280, have been demonstrated to alleviate digestive and neurological diseases. Furthermore, they have been shown to have great potential in the field of medicinal food and health food (Liu et al., 2023).

β-Eudesmol (CA323) and β-vetivenene (CA405) share a similar structure. CA323, less distributed and present at lower levels in CP, belongs to the eudesmane-type sesquiterpene with a decahydronaphthalene skeleton. Germacrene serves as a key biosynthetic intermediate, leading to protonation and secondary cyclization that produces eudesmane and eudesmol (Lu et al., 2023). CA405 was found in abundance in CP and is categorized as an eremophilane-type sesquiterpenoid, known for its antitumor, antimicrobial and anti-inflammatory properties. It is detected for the first time in CP and is widespread in certain genera of the Asteraceae family (Wu et al., 2016). Azulene (CA161) is a unique sesquiterpenoid with aromatic hydrocarbon characteristics, composed of five- and seven-membered rings. It exhibits antibacterial and insecticidal activities, and is also used in the production of natural and safe insecticides (Akram et al., 2023). Additionally, there are guaiane-type sesquiterpenoid: δ-guaiene CA464, as well as 2 unknown-type: (E)-sesquisabinene hydrate (CA274) and (Z)-sesquisabinene hydrate (CA463). These constituents are less abundant and not considered signature components in CP (Zhao et al., 2023).

Several tricyclic sesquiterpenoids have been detected in CP, including copaene-type sesquiterpenoid: α-copaene (CA237) and cis-β-copaene (CA250); cubebane-type sesquiterpenoid: α-cubebene (CA236) and (+)-β-cedrene (CA522); aromadendrane-type sesquiterpenoid: ledol (CA297); cedrene-type sesquiterpenoid: epicedrol (CA311), (3R,3aR,3bR,4S,7R,7aR)-4-Isopropyl-3,7-dimethyloctahydro-1H-cyclopenta[1,3]cyclopropa[1,2]benzen-3-ol (CA538) and bourbonene-type sesquiterpenoid: β-bourbonene (CA461). However, these constituents are less abundant and not considered signature components in CP.

3.2.3. Other terpenoids

In continuation of our detailed discussion on ring-structured sesquiterpenoids, CP also contains one acyclic sesquiterpene (E)-β-famesene (CA264) and two acyclic sesquiterpene esters. (E)-farnesyl acetate (CA226) and nerolidyl acetate (CA286) (Fig. S5). In this study, terpenoids were classified as sesquiterpene esters, a categorization theoretically justifiable from synthetic biology considerations. Acetyltransferase (ATF1) catalyzes the esterification of farnesol (FOH) and acetyl-CoA, resulting in the formation of farnesyl acetate. This hypothesis suggests that CA226 could be synthetically produced from CA264, despite the absence of corresponding sesquiterpene alcohols in CP (Guo, Kong, et al., 2018). CP essential oil contained two chain diterpene alcohols, two chain triterpenes and one cyclic triterpenoid. Squalene (CA345) and its isomer trans-squalene (CA451) can be synthesized from FPP via squalene synthase catalysis. In addition, FPP serves as a substrate for the synthesis of geranylgeraniol (CA346) and the earlier-mentioned FOH substrates. Squalene also can generate 2,3-oxidosqualene (CA534) through the action of squalene epoxidase. However, these compounds are not widely distributed and are relatively less abundant in CP; hence, they will not be studied extensively.

3.3. Mechanism and functions of other 10 types of VOCs in CP

3.3.1. Alkanes, alkenes and alicyclic hydrocarbons

Hydrocarbon compounds detected in CP included alkanes, alkenes, alicyclic hydrocarbons and alkynes, and as only one type of alkynes was detected, they were classified in other categories and will not be discussed in detail. Alkanes, with their wide variety of compounds, significantly influence the growth and development of CACH (Fig. S6). CP is abundant in long-chain alkanes, such as pentacosane (CA330), nonacosane (CA358) and dotriacontane (CA410). These alkanes, which are found in the epidermal cuticular wax of citrus fruits, constitute the initial barrier to interaction with the external environment. They play a crucial role in preventing water loss, regulating temperature fluctuations, diffusing gases, protecting against pathogens, and determining fruit firmness. Furthermore, a reduction in the proportion of alkanes and terpenoids in the cuticular wax of fruit after harvest is associated with increased cuticle permeability and fruit water loss (Romero & Lafuente, 2022). Alkenes (including terpenoids) are considered the primary VOCs and aroma-active constituents of citrus fruits (Fig. S7). In a study by Wang et al., 2022 on the aroma-active constituents in citrus-white tea, D-limonene, γ-terpinene, β-myrcene and β-pinene were found to have citrus-like herbaceous and greenish odors. Furthermore, citrus fruits are rich in terpene aromas and precursors, and the addition of citrus pericarp to the preparation of tea and other beverages can enhance the aroma quality and flavor of blended beverages. The functional activity of alkenes in CP has been the subject of numerous studies, with a particular focus on terpenes and terpenoids. These have been discussed and analyzed in detail above. The alicyclic hydrocarbons detected in CP predominantly consisted of five- and six-membered rings (Fig. S8). There are fewer scientific studies on alicyclic hydrocarbons in CP, and the distribution of their contents was limited, so no in-depth study has been undertaken.

3.3.2. Aromatic compounds

The benzene ring-containing compounds detected in CP exhibit diversity and complexity in classification. Consequently, all compounds featuring structural benzene rings were classified as aromatic compounds and ranked according to the type and number of functional groups (Fig. S9). Studies have confirmed that oxygenated aromatic compounds, including phenols, phenol ethers, aldehydes and aromatic acids, have potent bacteriostatic effects against citrus sour rot pathogens. For instance, carvacrol (CA199) has been employed as a natural antimicrobial and preservative for food preservation and conservation (Che et al., 2022).
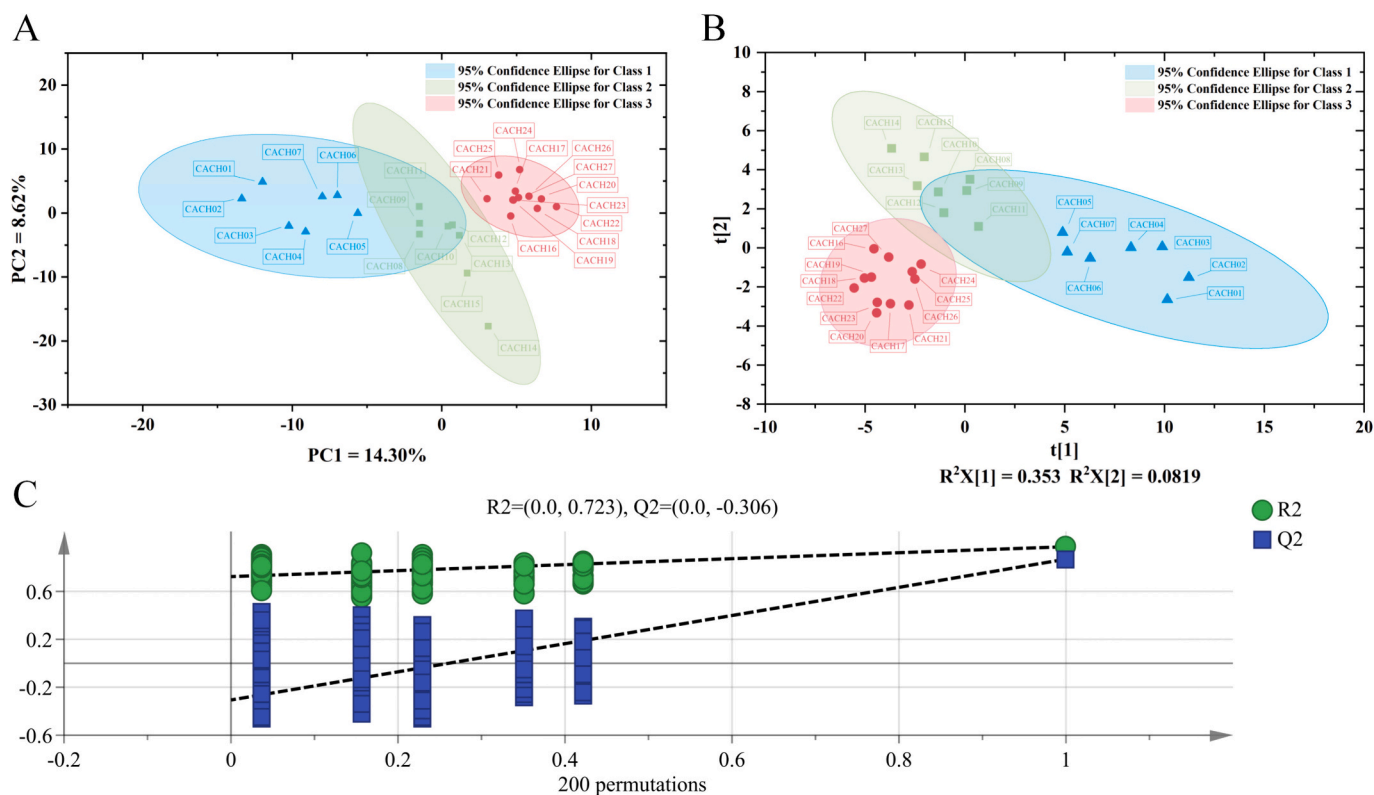
Fig. 4. Multivariate analysis of CP with SIMCA-P. (A) PCA score plots with pareto scaling mode. (B) PLS-DA score plots with pareto scaling mode ($R^2X = 0.541$, $R^2Y = 0.963$, and $Q^2 = 797$). (C) Crossing-validation with 200 time of calculations by using permutation test ($R^2 = 0.723$ and $Q^2 = -0.306$).

3.3.3. Alcohols and ethers

A total of 68 alcohols were detected in CP, constituting the second-highest number of species after alkanes and terpenes (Fig. S10). In this study, terpenoids are classified as oxygenated alcohols, such as linalool (CA118) and $(-)$-α-terpineol (CA167) mentioned above. CA118 exhibits citrus fruit and rose aromas, and can be degraded to CA167 and terpinene-4-ol during heating, indicating that processing may influence the flavor profile of related citrus beverages (Wang et al., 2022). While, it has been demonstrated that other fatty alcohols may be associated with the synthesis of esters in CP (Ribeaucourt et al., 2022). Ether compounds in CP exhibit low distribution and relative content, with the majority being mixed ethers and cyclic ether epoxides (Fig. S11). While ethers are common in citrus essential oils, they have not been subjected to investigation as potential aroma-active components and consequently, are not the focus of detailed investigation.

3.3.4. Aldehydes

The aldehydes detected in CP were roughly classified based on carbon chain length and unsaturated bonds (Fig. S12). Short-chain fatty aldehydes have been a key ingredient in food additives and perfumes since ancient times and occupy a central position in the field of flavors and fragrances. Fatty aldehydes $\geq$ C6 display a dual "citrus-like/fatty" character. Even-numbered fatty aldehydes are naturally present in the oil glands of citrus pericarps and impart a green, fresh, and citrusy flavor. Short-chain fatty aldehydes present in CP include octanal (CA077), nonanal (CA121), decanal (CA168) and dodecanal (CA169). They exhibit a diverse range of odors, such as citrus fruity, floral and grassy fruity (Ribeaucourt et al., 2022).

3.3.5. Ketones

Ketones detected in CP were primarily 2-methyl-4-pentanal (CA053), 2,5-dimethyl-3-hexanone (CA200), methyl(2,2-dimethylcyclohexyl) ketone (CA208) and 4-tert-butylcyclohexanone

(CA312). These ketones are likely to have been formed by the long-chain variants through the oxidation and degradation of fatty acids (Fig. S13) (Liu et al., 2022). Two monoterpene ketones were also identified, namely $(-)$-carvone (CA187) and $(+)$-carvone (CA188). Both compounds are known for their antioxidant and anti-inflammatory effects (Pina et al., 2022).

3.3.6. Fatty acids

Fatty acids constitute the main components of waxes in the citrus pericarp cuticle, such as (Z,Z)-9,12-octadecadienoic acid (CA360), octadecanoic acid (CA394) and pentadecanoic acid (CA395), which play a crucial role in wax composition (Fig. S14). During the postharvest storage of citrus fruits, the accumulation of fatty acids is promoted by low humidity storage conditions, which in turn leads to increased cuticle permeability (Liu et al., 2023). However, various factors, including citrus cultivar and growth environment, contribute to the lower presence of fatty acids in CP. Only n-hexadecanoic acid (CA363) and 2,6,11,15-tetramethylhexadecane (CA396) were consistently detected in CP with high relative contents. These compounds play a role in the protection and preservation of freshness during the growth and development of CACH.

3.3.7. Esters

Esters represent a pivotal class of volatiles in CP (Fig. S15), with high accumulation occurring during the late fruit-picking period. These esters may play a substantial role in the aroma of maturing fruits in CP (Richter et al., 2015). The production of esters in CACH is contingent upon the availability of acyl-CoA and alcohols. Alcohol acyltransferases possess the capacity to bind various alcohols and acyl-CoA, thereby enabling the synthesis of a diverse array of esters (Liu et al., 2022). The esters identified in CACH are also present in other citrus species, including (Z)-neryl acetate (CA227), linalyl acetate (CA189), and (E)-neryl acetate (CA192).

Table 2
VOCs with VIP > 1 in CP.

Number	Name	Category	VIP
CA016	2-Hexanol	Alcohols	1.30904
CA017	2,4-Dimethylheptane	Alkanes	1.95230
CA049	α-Pinene	Terpenoids	1.05138
CA071	(−)-β-Pinene	Terpenoids	2.02484
CA073	β-Myrcene	Terpenoids	1.44070
CA074	Tridecane	Alkanes	4.77455
CA077	Octanal	Aldehydes	1.25215
CA082	Dodecane	Alkanes	2.48679
CA083	5-Methyldecane	Alkanes	1.09632
CA089	o-Cymene	Aromatic compounds	1.47889
CA090	ᴅ-Limonene	Terpenoids	8.23139
CA095	(Z)-β-Ocimene	Terpenoids	1.29076
CA096	2,6,11-Trimethyldodecane	Alkanes	1.44039
CA098	5-Methylundecane	Alkanes	2.59578
CA099	γ-Terpinene	Terpenoids	3.83239
CA118	Linalool	Terpenoids	1.82514
CA158	2,6-Dimethylundecane	Alkanes	1.43444
CA194	4,6-Dimethyldodecane	Alkanes	1.03832
CA203	8-Methylheptadecane	Alkanes	1.27453
CA205	2-Methyldodecane	Alkanes	1.06924
CA228	1-Pentadecanal	Aldehydes	1.01876
CA234	Octadecane	Alkanes	1.59593
CA244	Tetradecane	Alkanes	2.01571
CA250	cis-β-Copaene	Terpenoids	1.04200
CA252	Germacrene B	Terpenoids	1.31127
CA257	δ-Cadinol	Terpenoids	1.35090
CA263	Octadecylcyclohexane	Alicyclic hydrocarbons	1.05010
CA264	(E)-β-Famesene	Terpenoids	1.63404
CA272	Germacrene D	Terpenoids	1.33548
CA276	3,5-Di-tert-butylphenol	Aromatic compounds	2.15154
CA277	2,4-Di-tert-butylphenol	Aromatic compounds	2.53923
CA324	9-Methylnonadecane	Alkanes	1.34643
CA330	Pentacosane	Alkanes	1.17799
CA349	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	Aromatic compounds	1.15300
CA358	Nonacosane	Alkanes	1.22651
CA359	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	Esters	2.75903
CA363	n-Hexadecanoic acid	Fatty acids	1.69823
CA383	5-Methoxypsoralen	Aromatic compounds	1.94945
CA396	Eicosanoic acid	Fatty acids	1.21813
CA400	1,4-Di-tert-pentylbenzene	Aromatic compounds	1.39966
CA405	β-Vetivenene	Terpenoids	2.03362
CA410	Dotriacontane	Alkanes	1.03427
CA428	2,2′-Methylenebis(6-t-butyl-4-methylphenol)	Aromatic compounds	1.07648
CA469	Linalyl 3-methylbutanoate	Terpenoids	1.42456
CA503	Octadecanal	Aldehydes	1.55008

3.4. Analysis of the characteristics of the VOCs in CP

3.4.1. Multivariate analysis of VOCs in CP

To evaluate VOCs variation in CP across distinct growth stages, this study employed PCA and PLS-DA to visualize the distribution of 544 VOCs and investigate the differences in total metabolites and intergroup metabolite differences among the various sample groups. The PCA score plot (Fig. 4A) displayed the first principal component (PC1) and the second principal component (PC2) scores on the horizontal and vertical coordinates. PC1 and PC2 contributed 14.30% and 8.62%, with a cumulative contribution of 22.92%. Based on the CACH harvesting period and PCA clustering results, the sample groups were divided into three distinct groups (Class 1 in blue, Class 2 in green and Class 3 in red), and all sample groups had confidence intervals within 95%. Class 1 and Class 3 were significantly separated, signifying a significant difference in VOCs. Conversely, Class 2 overlapped with both Class 1 and Class 3,

suggesting greater similarity in VOCs between Class 2 and Class 1. The three sample groups exhibited a significant difference in terms of PC1. Class 3 showed a positive correlation with Class 1, while Class 3 exhibited a positive correlation on PC1, and Class 1 showed a negative correlation on PC1. Meanwhile, Class 2 demonstrated a substantial intergroup difference on PC2, while Class 3 displayed the smallest intergroup difference. This suggested a high degree of similarity in VOCs of CP samples at this stage, indicating minimal differences when collecting CP for essential oil extraction. PLS-DA can identify differential metabolites by maximizing intergroup differences. Fig. 4B showed the PLS-DA score plots. Model parameters ($R^2X = 0.541$, $R^2Y = 0.963$, and $Q^2 = 797$) indicated that the model effectively explained a high level of variance (R^2Y) and exhibited strong cross-validated predictive power (Q^2). Furthermore, the model did not overfit, as evidenced by the execution of 200 iterations of cross-validation with low intercepts ($R^2 = 0.723$ and $Q^2 = -0.306$) (Fig. 4C). This suggested that the model can be used for further screening of differential metabolites. The score plots demonstrated that the three sample groups were not entirely separated, with a high degree of aggregation in Class 1 and a discrete group in Class 3, aligning with the PCA results.

3.4.2. Analysis of key VOCs in CP

PLS-DA screened for discriminative metabolites, and variables important in projection (VIP) values were used to identify metabolites significantly contributing to sample discrimination. Compounds with VIP > 1.0 were generally regarded as substantial contributors to the flavor of CP. Out of 544 VOCs, 45 with VIP > 1.0 were identified (Table 2, Fig. S16). The study examined the 45 key VOCs (Fig. S17). The pie chart shows that the main VOCs with VIP > 1.0 were alkanes (16 species) and terpenoids (14 species), followed by aromatic compounds (7 species). Additionally, three aldehydes, two fatty acids, and one each of alicyclic hydrocarbons, esters, and alcohols were screened. The following compounds with VIP values (> 2.0), significantly impacted the aroma of CP: (−)-β-pinene (CA071), tridecane (CA074), dodecane (CA082), ᴅ-limonene (CA090), 5-methylundecane (CA098), γ-terpinene (CA099), tetradecane (CA244), 3,5-di-tert-butylphenol (CA276), 2,4-ditert-butylphenol (CA277), 7,9-ditert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-d (CA359) and β-vetivenene (CA405).

All VOCs and their relative contents in CP were clustered and analyzed using GC–MS results (Fig. S18). Because of the excessive number of detected VOCs in CP in this study, most were sparsely distributed with low relative contents. Thus, only the clustering of the 45 key VOCs in CP among the sample groups was examined, highlighting changes in relative content and distribution characteristics through a heat map (Fig. 5). The blue area indicated lower relative content, and the red area showed higher relative content, revealing a distinct difference in the intensity of the 45 key VOCs in CP. Examining the clustering relationships of the samples reveals a strong correlation within Class 1, particularly between CACH01–04 and CACH05–07. The correlation between Class 2 and Class 1 is lower, while Class 3 exhibits a higher correlation with both Class 1 and Class 2. This is likely due to the higher number of samples in Class 3 and the gradual stabilization of the types and relative levels of the main VOCs as the CACH continued to develop and mature. Fourteen terpenoids with VIP > 1.0 were identified, including α-pinene (CA049), (−)-β-pinene (CA071), (Z)-β-ocimene (CA095), γ-terpinene (CA099), which were more abundant in CACH03 than in the other groups. The relative contents of linalool (CA118) and β-vetivenene (CA405) were highest in CACH02, and CACH06 had the highest relative content. The relative contents of these six terpenoids in Class 1 were significantly higher than those in Class 2 and Class 3, suggesting they are signature compounds of the initial stage of CACH growth and development. The relative content of ᴅ-limonene (CA090) and (E)-β-famesene (CA264) in CACH14 was much higher than in other samples. The expression of CA090 was notably higher than in Class 2 and Class 3, confirming once again that CA090 is less abundant in the predevelopment stage of CACH and gradually becomes the main
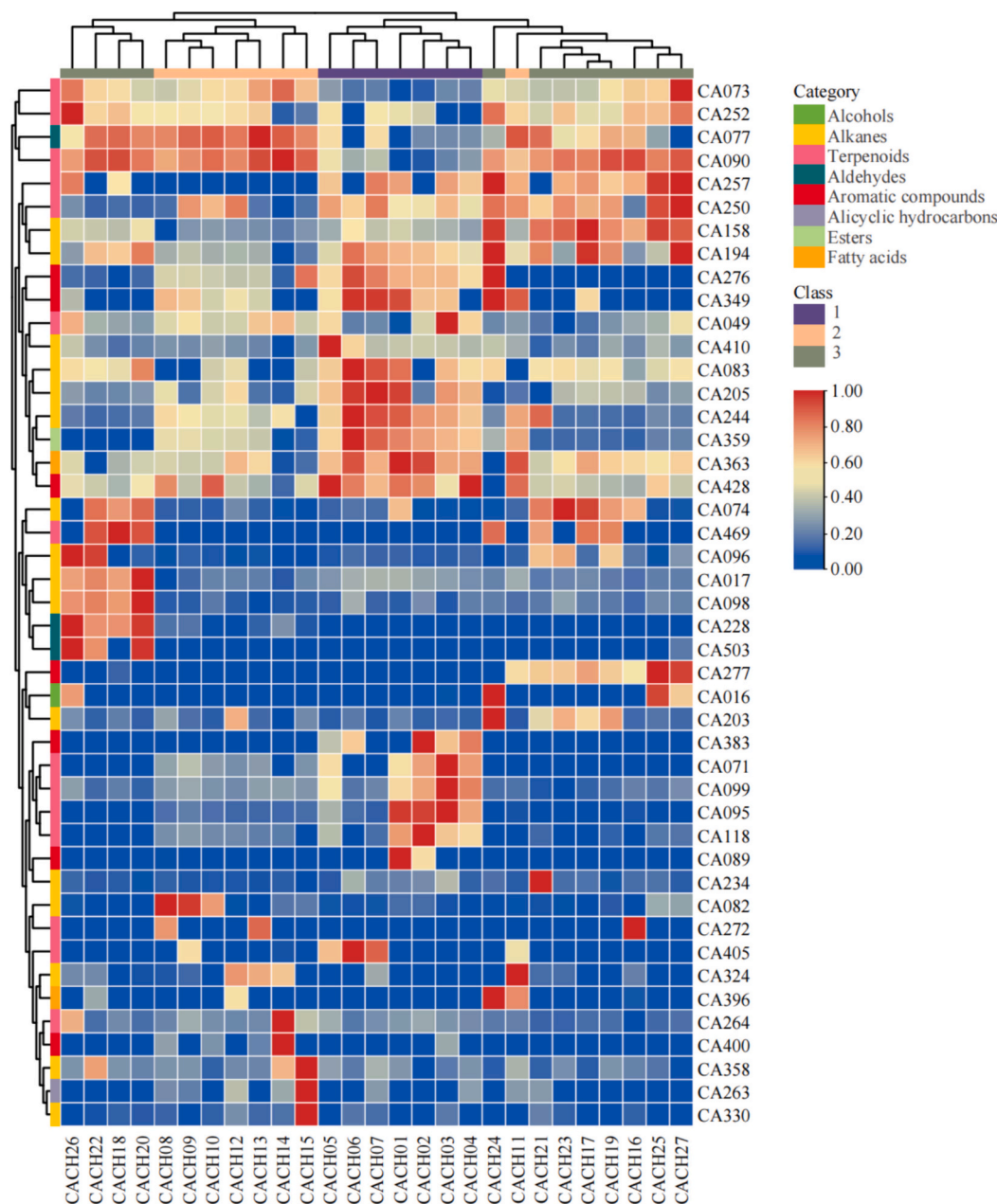
Fig. 5. Cluster heatmap analysis of VOCs with VIP > 1 in CP based on PLS-DS.

compound of the VOCs in CP as the fruit ripens (Hou et al., 2020). In addition, the relative levels of certain alkanes, esters, fatty acids and esters like 5-methyldecane (CA083), 2-methyldodecane (CA205), tetradecane (CA244), 7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (CA359), *n*-hexadecanoic acid (CA363) and 2,2′-methylenebis(6-t-butyl-4-methylphenol) (CA428) were markedly higher in Class 1 than in Classes 2 and 3.

3.5. Differential volatile metabolites analysis

The volcano plots (Fig. 6A-C) generated from *U* tests *p*-values and fold changes (FC) of metabolites offer rapid insights into variations in VOC expression levels and the statistical significance of differences among sample groups (Cao et al., 2022). Comparisons of VOCs relative levels in CP samples at distinct growth stages, using conditions of *p*-value >1, FC > 2 or FC < 0.5 were conducted to identify different volatile metabolites. The results revealed 24 VOCs with significant

differences between Class 1 and Class 2 (7 up-regulated and 17 down-regulated) (Fig. 6A); 37 significantly different VOCs between Class 2 and Class 3 (13 up-regulated and 24 down-regulated) (Fig. 6B); 46 significantly different VOCs between Class 1 and Class 3 (11 up-regulated and 35 down-regulated) (Fig. 6C). The fold change diagram was employed to illustrate significant changes in differential volatile metabolites between groups after the difference fold $\log_2$ treatment (Fig. 6D-F). The results indicated that in the early stage of CACH development (Class 1 vs. Class 2), up-regulated differentially volatile metabolites were mainly terpenoids, such as (*Z*)-neryl acetate (CA227) ($\log_2$(FC) = 2.43), while down-regulated differentially volatile metabolites were mainly alkanes, including 17-pentatriacontene (CA207) ($\log_2$(FC) = −2.48). At the later stages of CACH growth and development (Class 2 vs. Class 3), up-regulated differential volatile metabolites were predominantly alkanes, notably 2,6,11-trimethyldodecane (CA096) ($\log_2$(FC) = 3.84), and down-regulated differential volatile metabolites were chiefly alkanes and terpenoids, with the highest
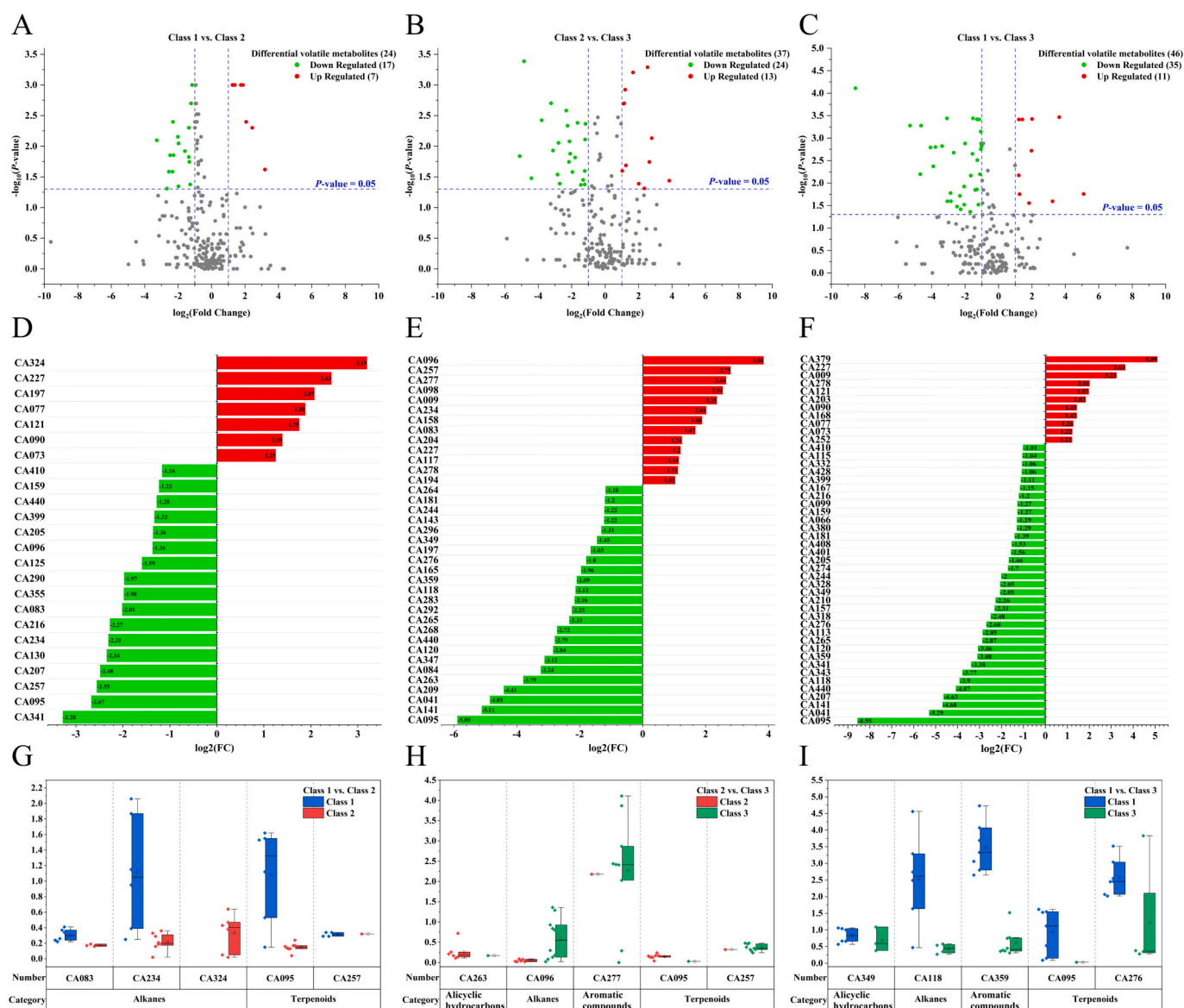
Fig. 6. Differential volatile metabolites between CP classes at different growth stages. (A-C) Volcano plots of the 544 identified VOCs. Differential metabolites were defined as metabolites with *p*-value <0.05 and abs log$_2$(FC) > 1. (D—F) plot of FC of differential volatile metabolites in each class, where the horizontal coordinate indicates log$_2$(FC) and the vertical coordinate indicates the number of CP differential VOCs. (G-I) Box plots of further screened groups of significantly different metabolites.

multiplicative down-regulation observed in (Z)-β-ocimene (CA095) (log$_2$(FC) = −5.89). Considering the entire harvest period (Class 1 vs. Class 3), the highest number of differential volatile metabolites were screened, and the up- and down-regulated differential volatile metabolites were mainly alkanes and terpenes. The most pronounced up-regulation was observed in 2-methylheptadecane (CA379) (log$_2$(FC) = 5.10), and the most substantial down-regulation in CA095 (log$_2$(FC) = −8.55). These VOCs can serve as potential differential metabolites between groups. Monitoring the changes in volatile metabolite abundance at different stages revealed that the FC of CA227 consistently exceeded 1 throughout the harvesting period, whereas D-limonene (CA090), a signature compound in CP, was only up-regulated in the early stage of growth. This indicates a significant increase in the relative expression of CA090 during the early development of CACH, with no significant change in the late development stage of CACH.

To further identify the CP differential volatiles during different growth stages, FCs were ranked within the range of *p*-value <0.05 and VIP > 1 (the larger the FC, the higher the significance level of the difference). The 15 markers identified have been presented in boxplots. The analysis revealed 11, 16 and 15 differential metabolites with VIP > 1 between Class 1 vs. Class 2, Class 2 vs. Class 3 and Class 1 vs. Class 3, respectively. Significant differences were observed in the relative content of octadecane (CA234) and (Z)-β-ocimene (CA095) between Class 1 and Class 2. Additionally, 9-methylnonadecane (CA324) was undetected in Class 1, and δ-cadinol (CA257) was detected only once in Class 2 (Fig. 6G). Significant differences were found in the relative content of 2,6,11-trimethyldodecane (CA096) and the distribution of CA095, CA257, octadecylcyclohexane (CA263), 2,4-di-tert-butylphenol (CA277) between Class 2 and Class 3 (Fig. 6H). Moreover, the differences in volatile metabolites were more pronounced in Class 1 vs. Class 3 (Fig. 6I), and the five screened different VOCs in Class 1 exhibited higher levels than those in Class 3, indicating a more significant decrease in the relative content of certain VOCs in CP after CACH maturation.

4. Conclusions

This study identified 544 VOCs in CP throughout the harvest period and categorized and summarized them. It was observed that the VOCs present in CP exhibited notable dissimilarities during the initial growth and development stages. However, as CACH matured, the divergence in VOCs diminished and eventually stabilized, as illustrated by Venn diagrams. Preliminary analyses were conducted on the relative content and distribution of VOCs at different growth stages and for different types were carried out. The changes in alkanes and terpenes were particularly noteworthy. As terpenoids constitute the primary bioactive substances in CP, our focus was on them. The study delved into the taxonomic situation structure and distribution characteristics of terpenoids in CP, with a particular focus on their diverse applications in the food industry and food science and technology. Additionally, it provided a preliminary assessment of the biosynthesis pathways of the monoterpenoids and sesquiterpenoids detected in CP. Subsequently, metabolomics methods were employed to analyze and screen differential metabolites in CP at multiple levels. The results unveiled 24, 37, and 46 differential metabolites between Class 1 vs. Class 2, Class 2 vs. Class 3, and Class 1 vs. Class 3, respectively. Among these, 11, 16, and 15 had VIP > 1, with the majority being terpenoids and alkanes. This study enhanced the compositional information on natural VOCs in CP and citrus, as well as references for their potential applications in the food industry. Additionally, it furnished a scientific basis for identifying differential metabolites in *C. aurantium* Changshan-huyou pericarp at different growth stages, aiding in the further research and development of *C. aurantium* Changshan-huyou.

CRediT authorship contribution statement

Qi Cui: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Li-Jie Jiang:** Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation. **Le-Le Wen:** Visualization, Investigation, Formal analysis. **Xiao-Li Tian:** Visualization, Resources, Formal analysis. **Qiang Yuan:** Supervision. **Ju-Zhao Liu:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101631.

References

Akram, W., Tagde, P., Ahmed, S., Arora, S., Emran, T. B., Babalghith, A. O., … Simal-Gandara, J. (2023). Guaiazulene and related compounds: A review of current perspective on biomedical applications. *Life Sciences, 316*, Article 121389.

Alsharif, A., Kozhevnikova, E. F., & Kozhevnikov, I. V. (2023). Selective dehydroisomerization of cyclic monoterpenes to p-cymene over silica-supported CdO. *Applied Catalysis B: Environmental, 325*, Article 122362.

Bai, X. D., & Tang, J. (2020). Myrcene exhibits antitumor activity against lung cancer cells by inducing oxidative stress and apoptosis mechanisms. *Natural Product Communications, 15*(9), Article 1934578X20961189.

Beran, F., Köllner, T. G., Gershenzon, J., & Tholl, D. (2019). Chemical convergence between plants and insects: Biosynthetic origins and functions of common secondary metabolites. *The New Phytologist, 223*(1), 52–67.

Bergman, M. E., Franks, A. E., & Phillips, M. A. (2023). Biosynthesis, natural distribution, and biological activities of acyclic monoterpenes and their derivatives. *Phytochemistry Reviews, 22*(2), 361–384.

Cao, X. D., Ru, S. J., Fang, X. G., Li, Y., Wang, T. Y., & Lyu, X. M. (2022). Effects of alcoholic fermentation on the non-volatile and volatile compounds in grapefruit (*Citrus paradisi* mac. cv. Cocktail) juice: A combination of UPLC-MS/MS and gas chromatography ion mobility spectrometry analysis. *Frontiers in Nutrition, 9*, Article 1015924.

Che, J. X., Pan, F., Chen, X. M., Zhang, Y. H., Tao, N. G., & Fu, Y. S. (2022). Screening of oxygenated aromatic compounds for potential antifungal activity against *Geotrichum citri-auranti* through structure-activity relationship analysis. *Journal of Agricultural and Food Chemistry, 70*(42), 13787–13795.

Chen, C. J., Wu, Y., Li, J. W., Wang, X., Zeng, Z. H., Xu, J., … Xia, R. (2023). TBtools-II: A "one for all, all for one" bioinformatics platform for biological big-data mining. *Molecular Plant, 16*(11), 1733–1742.

Chen, J. (2023). Essential role of medicine and food homology in health and wellness. *Chinese Herbal Medicines, 15*(3), 347–348.

Chen, Y., Zhu, Z. P., Chen, J., Zheng, Y. F., Limsila, B., Lu, M. G., … Liao, W. (2021). Terpenoids from *Curcumae Rhizoma*: Their anticancer effects and clinical uses on combination and versus drug therapies. *Biomedicine & Pharmacotherapy, 138*, Article 111350.

Falleh, H., Ben Jemaa, M., Djebali, K., Abid, S., Saada, M., & Ksouri, R. (2019). Application of the mixture design for optimum antimicrobial activity: Combined treatment of *Syzygium aromaticum*, *Cinnamomum zeylanicum*, *Myrtus communis*, and *Lavandula stoechas* essential oils against *Escherichia coli*. *Journal of Food Processing and Preservation, 43*(12), Article e14257.

Feng, G. Z., Wu, J. X., Xu, Y. H., Lu, L. Q., & Yi, H. L. (2021). High-spatiotemporal-resolution transcriptomes provide insights into fruit development and ripening in *Citrus sinensis*. *Plant Biotechnology Journal, 19*(7), 1337–1353.

Food and Agriculture Organization of the United Nations. (2022). FAOSTAT Database-Resources. https://www.fao.org/faostat/en/#data.

Gao, L., Zhang, H., Yuan, C. H., Zeng, L. H., Xiang, Z., Song, J. F., … Jiang, J. P. (2022). *Citrus aurantium* "Changshan-huyou"-an ethnopharmacological and phytochemical review. *Frontiers in Pharmacology, 13*, Article 983470.

Guo, D., Kong, S., Zhang, L., Pan, H., Wang, C., & Liu, Z. (2018). Biosynthesis of advanced biofuel farnesyl acetate using engineered *Escherichia coli*. *Bioresource Technology, 269*, 577–580.

Guo, J. J., Gao, Z. P., Li, G. Y., Fu, F. H., Liang, Z. E., Zhu, H., & Shan, Y. (2019). Antimicrobial and antibiofilm efficacy and mechanism of essential oil from *Citrus Changshan-huyou* Y. B. Chang against *listeria monocytogenes*. *Food Control, 105*, 256–264.

Guo, J. J., Gao, Z. P., Xia, J. L., Ritenour, M. A., Li, G. Y., & Shan, Y. (2018). Comparative analysis of chemical composition, antimicrobial and antioxidant activity of citrus essential oils from the main cultivated varieties in China. *LWT Food Science and Technology, 97*, 825–839.

Hao, D., & Liu, C. (2023). Deepening insights into food and medicine continuum within the context of pharmacophylogeny. *Chinese Herbal Medicines, 15*(1), 1–2.

Harms, V., Schröder, B., Oberhauser, C., Tran, C. D., Winkler, S., Dräger, G., & Kirschning, A. (2020). Methyl-shifted farnesyldiphosphate derivatives are substrates for sesquiterpene cyclases. *Organic Letters, 22*(11), 4360–4365.

Hashiesh, H. M., Sharma, C., Goyal, S. N., Sadek, B., Jha, N. K., Kaabi, J. A., & Ojha, S. (2021). A focused review on CB2 receptor-selective pharmacological properties and therapeutic potential of β-caryophyllene, a dietary cannabinoid. *Biomedicine & Pharmacotherapy, 140*, Article 111639.

Hou, J. X., Liang, L., & Wang, Y. X. (2020). Volatile composition changes in navel orange at different growth stages by HS-SPME-GC-MS. *Food Research International, 136*, Article 109333.

Jiang, H., & Wang, X. (2023). Biosynthesis of monoterpenoid and sesquiterpenoid as natural flavors and fragrances. *Biotechnology Advances, 65*, Article 108151.

Jiang, L. J., Tian, X. L., Yuan, Q., Liu, J. Z., & Cui, Q. (2022). Research progress on cultivation techniques of *Citrus × aurantium* "Changshanhuyou" and chemical components and pharmacological action of Qu Aurantii Fructus. *Journal of Zhejiang Forestry Science and Technology, 42*(5), 129–136.

Juárez, Z. N., Bach, H., Sánchez-Arreola, E., Bach, H., & Hernández, L. R. (2016). Protective antifungal activity of essential oils extracted from *Buddleja perfoliata* and *Pelargonium graveolens* against fungi isolated from stored grains. *Journal of Applied Microbiology, 120*(5), 1264–1270.

Kim, D. S., Lee, S., Park, S. M., Yun, S. H., Gab, H. S., Kim, S. S., & Kim, H. J. (2021). Comparative metabolomics analysis of citrus varieties. *Foods, 10*(11), 2826.

Kim, H., Srividya, N., Lange, I., Huchala, E. W., Ginovska, B., Lange, B. M., & Raugei, S. (2022). Determinants of selectivity for the formation of monocyclic and bicyclic products in monoterpene synthases. *ACS Catalysis, 12*(12), 7453–7469.

Kvittingen, L., Sjursnes, B. J., & Schmid, R. (2021). Limonene in citrus: A string of unchecked literature citings? *Journal of Chemical Education, 98*(11), 3600–3607.

Lei, D., Qiu, Z., Qiao, J., & Zhao, G. R. (2021). Plasticity engineering of plant monoterpene synthases and application for microbial production of monoterpenoids. *Biotechnology for Biofuels, 14*(1), 147.

Li, Z. Z., Howell, K., Fang, Z. X., & Zhang, P. Z. (2020). Sesquiterpenes in grapes and wines: Occurrence, biosynthesis, functionality, and influence of winemaking processes. *Comprehensive Reviews in Food Science and Food Safety, 19*(1), 247–281.

Liu, H. C., Wen, J., Xu, Y. J., Wu, J. J., Yu, Y. S., Yang, J. G., … Fu, M. Q. (2022). Evaluation of dynamic changes and formation regularity in volatile flavor compounds in *Citrus reticulata* 'chachi' peel at different collection periods using gas chromatography-ion mobility spectrometry. *LWT Food Science and Technology, 171*, Article 114126.

Liu, Y. Q., Zhang, Z. X., Lin, W. L., Liang, H. X., Lin, M., Wang, J. L., … Zheng, Y. Z. (2023). A novel FCTF evaluation and prediction model for food efficacy based on association rule mining. *Frontiers in Nutrition, 10*, Article 1170084.

Lu, X. M., Zhao, C. Y., Shi, H., Liao, Y. C., Xu, F., Du, H. J., … Zheng, J. K. (2023). Nutrients and bioactives in citrus fruits: Different citrus varieties, fruit parts, and growth stages. *Critical Reviews in Food Science and Nutrition, 63*(14), 2018–2041.

Lu, X. P., Li, F. F., Xiong, J., Cao, X. J., Ma, X. C., Zhang, Z. M., … Xie, S. X. (2017). Transcriptome and metabolome analyses provide insights into the occurrence of peel roughing disorder on satsuma mandarin (*Citrus unshiu* Marc.) fruit. *Frontiers in Plant Science, 8*, Article 1907.

Masyita, A., Mustika Sari, R., Dwi Astuti, A., Yasir, B., Rahma Rumata, N., Emran, T. B., … Simal-Gandara, J. (2022). Terpenes and terpenoids as main bioactive compounds of essential oils, their roles in human health and potential application as natural food preservatives. *Food Chemistry: X, 13*, Article 100217.

Miao, W., Liu, X. J., Li, N., Bian, X. Q., Zhao, Y. R., He, J., … Wu, J. L. (2023). Polarity-extended composition profiling via LC-MS-based metabolomics approaches: A key to functional investigation of *Citrus aurantium* L. *Food Chemistry, 405*(Part B), Article 134988.

Nguyen, T. D., Kwon, M., Kim, S. U., Fischer, C., & Ro, D. K. (2019). Catalytic plasticity of germacrene a oxidase underlies sesquiterpene lactone diversification. *Plant Physiology, 181*(3), 945–960.

Pina, L. T. S., Serafini, M. R., Oliveira, M. A., Sampaio, L. A., Guimarães, J. O., & Guimarães, A. G. (2022). Carvone and its pharmacological activities: A systematic review. *Phytochemistry, 196*, Article 113080.

Ren, Y., Liu, S., Jin, G., Yang, X., & Zhou, Y. J. (2020). Microbial production of limonene and its derivatives: Achievements and perspectives. *Biotechnology Advances, 44*, Article 107628.

Ribeaucourt, D., Bissaro, B., Lambert, F., Lafond, M., & Berrin, J. G. (2022). Biocatalytic oxidation of fatty alcohols into aldehydes for the flavors and fragrances industry. *Biotechnology Advances, 56*, Article 107787.

Richter, A., Seidl-Adams, I., Köllner, T. G., Schaff, C., Tumlinson, J. H., & Degenhardt, J. (2015). A small, differentially regulated family of farnesyl diphosphate synthases in maize (*Zea mays*) provides farnesyl diphosphate for the biosynthesis of herbivore-induced sesquiterpenes. *Planta, 241*(6), 1351–1361.

Romero, P., & Lafuente, M. T. (2022). Relative humidity regimes modify epicuticular wax metabolism and fruit properties during navelate orange conservation in an ABA-dependent manner. *Food Chemistry, 369*, Article 130946.

Singh, B., Singh, J. P., Kaur, A., & Singh, N. (2020). Phenolic composition, antioxidant potential and health benefits of citrus peel. *Food Research International, 132*, Article 109114.

Singh, B., Singh, J. P., Kaur, A., & Yadav, M. P. (2021). Insights into the chemical composition and bioactivities of citrus peel essential oils. *Food Research International, 143*, Article 110231.

Staudt, A., Brack, Y., Itabaiana, I., Jr., & Leal, I. C. R. (2022). Biocatalytic synthesis of monoterpene esters–a review study on the phylogenetic evolution of biocatalysts. *Molecular Catalysis, 528*, Article 112464.

Tocmo, R., Pena-Fronteras, J., Calumba, K. F., Mendoza, M., & Johnson, J. J. (2020). Valorization of pomelo (*Citrus grandis* Osbeck) peel: A review of current utilization, phytochemistry, bioactivities, and mechanisms of action. *Comprehensive Reviews in Food Science and Food Safety, 19*(4), 1969–2012.

Tourabi, M., Nouioura, G., Touijer, H., Baghouz, A., el Ghouizi, A., Chebaibi, M., … Derwich, E. (2023). Antioxidant, antimicrobial, and insecticidal properties of chemically characterized essential oils extracted from *Mentha longifolia*: *In vitro* and *in silico* analysis. *Plants, 12*(21), 3783.

Wang, J. T., Shi, J., Zhu, Y., Ma, W. J., Yan, H., Shao, C. Y., … Lin, Z. (2022). Insights into crucial odourants dominating the characteristic flavour of citrus-white teas prepared from *Citrus reticulata* Blanco "Chachiensis" and *Camellia sinensis* "Fudingdabai". *Food Chemistry, 377*, Article 132048.

Wen, S., An, R., Li, D., Cao, J., Li, Z., Zhang, W., Chen, R., Li, Q., Lai, X., Sun, L., & Sun, S. (2022). Tea and *Citrus maxima* complex induces apoptosis of human liver cancer cells via PI3K/AKT/mTOR pathway *in vitro*. *Chinese Herbal Medicines, 14*(3), 449–458.

Wu, L., Liao, Z. X., Liu, C., Jia, H. Y., & Sun, J. Y. (2016). Eremophilane sesquiterpenes from the genus *Ligularia*. *Chemistry & Biodiversity, 13*(6), 645–671.

Xu, H., & Dickschat, J. S. (2023). Germacrene B - a central intermediate in sesquiterpene biosynthesis. *Beilstein Journal of Organic Chemistry, 19*, 186–203.

Yan, H., Pu, Z. J., Zhang, Z. Y., Zhou, G. S., Zou, D. Q., Guo, S., … Duan, J. A. (2021). Research on biomarkers of different growth periods and different drying processes of *Citrus wilsonii* Tanaka based on plant metabolomics. *Frontiers in Plant Science, 12*, Article 700367.

Zhang, Q. X., Song, W. Y., Tao, G. Q., Li, Q., Wang, L. X., Huang, W. K., … Ye, Y. P. (2023). Comparison of chemical compositions and antioxidant activities for the immature fruits of *Citrus changshan-huyou* Y.B. Chang and *Citrus aurantium* L. *Molecules, 28*(13), 5057.

Zhang, Q. Y., Feng, C., Li, W. H., Qu, Z. H., Zeng, M., & Xi, W. P. (2019). Transcriptional regulatory networks controlling taste and aroma quality of apricot (*Prunus armeniaca* L.) fruit during ripening. *BMC Genomics, 20*(1), 45.

Zhang, X. Y., Zhu, Z. J., Ren, S. P., Deng, Y. C., Xu, J. Y., Zhang, S. M., … Zhang, Q. (2022). Metabolomic navigated citrus waste repurposing to restore amino acids disorder in neural lesion. *Food Chemistry, 387*, Article 132933.

Zhao, Z. H., Hao, Y. F., Liu, Y. J., Shi, Y. S., Lin, X., Wang, L., … Li, J. X. (2023). Comprehensive evaluation of aroma and taste properties of different parts from the wampee fruit. *Food Chemistry: X, 19*, Article 100835.

Zhou, X. Y., Miao, J. Q., Zhang, B. R., Duan, M. X., Li, J. X., Yue, J. Q., … Cheng, Y. J. (2022). Cuticular wax metabolism of lemon (*Citrus Limon Burm*. f. Eureka) fruit in response to ethylene and gibberellic acid treatment. *Postharvest Biology and Technology, 194*, Article 112062.

Zielińska-Błajet, M., & Feder-Kubis, J. (2020). Monoterpenes and their derivatives-recent development in biological and medical applications. *International Journal of Molecular Sciences, 21*(19), 7078.