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Quality Deterioration and Volatile Metabolite Profile of 'Orah' Mandarin During Low-Temperature Storage and Room-Temperature Shelf Life

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Highlights

What are the main findings?

- The LTS should not exceed 100 days due to the intensified accumulation of off-flavor metabolites such as ethanol.
- Significant decrease in terpenes within the first 60 days of LTS resulted in fast loss of aroma, while the substantial increase in ethanol, methanol and acetaldehyde in the middle and later stages of LTS contributed to the prominent off-flavor.

What is the implication of the main findings?

- The resultant mechanistic framework will provide a robust theoretical basis for precision post-harvest management protocols specifically tailored for 'Orah' mandarin and reduce its loss.

Abstract

Low-temperature stored mandarin was prone to rapid quality deterioration when moved to a room-temperature shelf (RTS). However, systematic research on the safe threshold of time for low-temperature storage (LTS) and the key metabolic mechanisms underlying the quality deterioration was lacking. The dynamic changes in color, intrinsic quality, off-flavor and volatility of 'Orah' mandarin during LTS (6–8 °C, 0 d to 180 d) and thereafter RTS for 7 days were determined, and the key characteristic metabolites related to deterioration were analyzed. RTS after an LTS for 0 d, 20 d, 40 d, 60 d, 80 d, 100 d or 120 d exacerbated the decline in pericarp chromatic *a** value, content of soluble solid, titratable acid, vitamin C, sucrose and malic acid in the pulp, as well as the accumulation of ethanol, methanol and acetaldehyde. After the LTS for 120 days and thereafter RTS for 7 days, the accumulation of ethanol exceeded 2000 mg/L, resulting in a distinct unpleasant odor. Seven categories (alcohols, aldehydes, esters, lipids/fatty acyls, terpenoids, organic acids, tetrahydropyrans) and 43 volatile substances were detected in the pulp. A significant decrease in terpenes

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within the first 60 days of LTS resulted in fast loss of aroma, while the substantial increase in ethanol, methanol and acetaldehyde (ethyl octoate, caproic acid) in the middle and later stages of LTS contributed to the prominent off-flavor. In conclusion, the LTS should not exceed 100 days due to the intensified accumulation of ethanol. The above results indicated that intervention techniques should be implemented to inhibit the synthesis of ethanol in the middle of storage, and methods such as reducing temperature fluctuations should be used to block the rapid accumulation of ester compounds during the RTS.

Keywords: 'Orah' mandarin; low-temperature storage; room-temperature shelf; off-flavor; volatile metabolite profile

1. Introduction

'Orah' mandarin (*Citrus reticulata* × *C. sinensis* 'Orah'), which is characterized by tender pulp, abundant juice, and high nutritional value, had consequently become one of the pillar crops of the agricultural economy in Guangxi province in China. In 2024, its planted area in Guangxi province reached approximately 2.4 million mu (1.6×10^5 ha), and its yield was about 5.7 million tons, accounting for about 8% of the total citrus output in China. However, the rapid increase in production coupled with the simultaneous harvest and marketing of other fruits, has led to an increasing sale pressure for 'Orah' mandarin. Therefore, post-harvest storage is indispensable for solving this problem. Lin et al. demonstrated that 6–8 °C was the optimal storage temperature for maintaining the quality of 'Orah' mandarin [1]. Under the current logistical conditions, full cold-chain infrastructure was still unavailable in most distribution channels in China, and the quality changes of 'Orah' mandarin during long-term low-temperature storage (LTS) and transitions from LTS to room-temperature shelf (RTS) remained poorly investigated. Consequently, a systematic characterization of quality dynamics during LTS and subsequent retail RTS was urgently required.

Appropriate low-temperature storage is an important means to maintain the quality of fresh fruits and extend their supply. Minimal weight loss was observed in the strawberry fruit held at 3 °C and its firmness retained more than 70% after 7 days of LTS [2]. The sensory quality of 'W. Murcott' mandarin was not significantly lost when stored at 5 °C for 2 months [3]. No obvious chilling injury was found in grapefruit when kept at 11 °C and its accumulation of carotenoid and flavonoid increased along with the storage [4]. The peel color and water content of starfruit were preserved when stored at 4 °C with plastic wrapping for 6 days [5]. The custard apple (*Annona squamosa* L.) held at 10 °C showed suppressed respiration, retarded starch hydrolysis, sustained antioxidant enzyme activities and slower firmness loss [6]. More importantly, the fluctuations in temperature during storage, transportation and shelf life stages significantly affect the quality of the fruit. The peel chroma was retained and nutrient depletion was delayed when 'Huanghua' pear was stored at 1 °C, but its quality deteriorated and its marketability was rapidly lost within several days when transferred to an ambient shelf [7]. Anthocyanin content was retained and the loss of firmness, titratable acidity and vitamin C was slowed in blueberries when they were stored at 0 to 1 °C, but their ratio of commodity fruit dropped to 41% when transferred to 20 °C for 5 days [8]. The decay rate of table grapes was 2.4% when kept at -0.3 ± 0.7 °C for 14 d, but increased by 3.7% when exposed to 5.4 ± 0.6 °C for 10 d (simulated market conditions) [9]. Continuous 5 ± 0.1 °C throughout storage and transport enhanced the antioxidant capacity and maintained a high ATP energy charge and thus delayed the senescence of apple fruit [10]. Similarly, a small fluctuation in the temperature

in the storage facility and during the distribution process resulted in reduced accumulation of off-odor accumulation in 'Newhall' navel orange [11], whereas the elevated temperature during transport resulted in a higher decay rate, as well as a higher content of ethanol and acetaldehyde in 'Ponkan' mandarin [12].

Extensive research had been performed on the post-harvest physiology of citrus fruit, but quality changes in 'Orah' mandarin, especially the metabolic basis for the deterioration of sensory quality during the long-term low-temperature storage and the subsequent room-temperature storage, remained poorly characterized. This study completed a comprehensive time-course analysis to quantify changes in external quality attributes (weight loss, color indices), internal quality indicators (soluble solids, titratable acidity, ascorbic acid, predominant sugars and organic acids), fermentation catabolism (ethanol, acetaldehyde, methanol), and volatility profile. By integrating these data, we aimed to identify the critical metabolite factors and elucidate the underlying mechanisms driving the quality deterioration in 'Orah' mandarin during the RTS after LTS. The resultant mechanistic framework will provide a robust theoretical basis for precision post-harvest management protocols specifically tailored for 'Orah' mandarin and will reduce its loss.

2. Materials and Methods

2.1. Materials and Treatments

On 24 January 2022 (307 days after full bloom), uniform and defect-free 'Orah' mandarin (*Citrus reticulata* × *C. sinensis*) was hand-harvested at the Hongyuan Orchard (24°30′31″ north latitude, 110°57′38″ east longitude), Pingle County, Guilin, Guangxi, China. Harvested fruits were randomly divided into three groups, each of which contained 1000 fruits, and were transported to the laboratory within 2 h. Selected fruits were subjected to a standard commercial treatment by dipping with fungicide (0.04% pyrimethanil + 0.03% prochloraz + 0.02% 2,4-D) for 2 min, then air-dried in a shaded and well-ventilated area, individually wrapped in polyethylene bags (5 μm thickness; Xinjiang® high-pressure preservation bags, produced by Jiangxi Xinfeng Xinjiang Plastic Packaging Co., Ltd, Xinfeng, Jiangxi province, China), and placed in a refrigeration room at 6–8 °C (low-temperature storage, LTS) and 85% relative humidity. Sampling was performed at 0 (without LTS), 20, 40, 60, 80, 100, 120, 140, 160 and 180 days after harvest (DAH). At each time point, 10 fruits were randomly selected and used as one replicate, which was then juiced. The freshly extracted juice from each time point with three biological replicates was immediately used for determining total soluble solid (TSS), titratable acid (TA) and vitamin C (VC) (see Section 2.3), while juice from each time point with three biological replicates was immediately frozen at −40 °C until analysis of sugars and organic acids (see Section 2.4).

At 0 (without LTS), 20, 40, 60, 80, 100, and 120 DAS, 10 fruits used as one replicate were transferred from the refrigeration room to 20 °C for 7 days to test room-temperature shelf life (LTS + RTS). Three biological replicates were performed. Juice was then extracted and stored at −40 °C as above. Because strong off-odor was detected in fruit with LTS_120d + RTS_7d, the room-temperature shelf life of fruits transferred from LTS for more than 120 days was terminated. Methanol, ethanol and acetaldehyde, which were the predominant components of off-odor in the juice samples, were then analyzed (see Section 2.5).

2.2. Reagents

Analytical-grade sodium hydroxide (NaOH), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), 2,6-dichlorophenol-indophenol, phenolphthalein, anhydrous oxalic acid, ascorbic acid, hydroxylamine hydrochloride, pyridine, hexamethyldisilazane (HMDS), and trimethylchlorosilane (TMCS) were purchased from Sinopharm Chemical Reagent Co., Ltd.

(Shanghai, China). HPLC graded 4-methyl-2-pentanol, phosphoric acid, and acetonitrile were obtained from Merck KGaA (Darmstadt, Germany). HPLC-grade methanol, ethanol, acetaldehyde, malic acid, citric acid, fructose, glucose, and sucrose were supplied by Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Ultra-pure water was prepared by distilling water through a Milli–Q A10 system (Millipore, Milford, MA, USA).

2.3. Determination of Chromatic Values of Pericarp

The chromatic value *L** (lightness, 0 = black, 100 = white), *a** (+, red; –, green) and *b** (+, yellow; –, blue) of pericarp on the 6 equidistant equatorial sites (60° apart) of each fruit were measured three times using a colorimeter (CR-400, Konica Minolta, Japan). The measurement at each time point was subjected to 30 repetitions (fruits). The color index (CI) was calculated as follows:

$$CI = 1000\ a/Lb \quad (1)$$

2.4. Determination of TSS, TA and VC in Juice

TSS, TA and VC content was determined using freshly extracted juice (see Section 2.1). TSS content was measured by a brix refractometer (RA-250WE, Kyoto Electronics, Japan). TA content was quantified by an indicator-titration method with 0.1 mol L^{-1} NaOH to pH 8.1 (1% phenolphthalein) according to GB/T 8210-2011 (Method of inspection for fresh citrus fruit, China, 2011) and expressed as g citric acid per 100 mL juice (*w*/*v*, %). VC content was assayed by 2,6-dichlorophenol-indophenol titration according to a method described in Chinese food safety standards (GB 5009.86-2016, National food safety standard—Determination of ascorbic acid, China, 2016) and expressed as mg mL^{-1} juice.

2.5. Determination of Content of Sucrose, Glucose, Fructose, Malic Acid and Citric Acid

The content of sucrose, glucose, fructose, malic acid and citric acid were quantified following the protocol of Zhou et al. [13] with some modifications. Briefly, 5 mL of freshly extracted juice was transferred to a 25 mL tube, mixed with 15 mL ultra-pure water, and then incubated at 75 °C for 50 min. After being cooled, the mixture was centrifuged at 12,000× *g* for 15 min, and then 10 mL of the supernatant was pipetted to a volumetric flask and made up to 25 mL with ultra-pure water. The sample solution was filtered through a 0.22 μm membrane and a 10 μL sample was injected to a Shimadzu LC-20A system(Shimadzu, Suzhou, China).

For measuring sucrose, glucose and fructose, a Shimadzu LC-20A system was linked with a refractive-index detector (RID-20A) and an Inertsil NH_2 column (4.6 mm × 250 mm, 5 μm; column temperature, 40 °C) using the optimum mobile phase, which was composed of 75% acetonitrile (*v*/*v*) and 25% ultra-pure water (*v*/*v*). The flow rate was 1.0 mL min^{-1}.

For measuring malic acid and citric acid, the Shimadzu LC-20A system was linked with a UV detector (SPD-20A) at 210 nm, and a Inertsil ODS-3 C_{18} column (4.6 mm × 250 mm, 5 μm; column temperature, 17 °C) using a mobile phase composed of acetonitrile (4%, *v*/*v*) and 0.02% phosphoric acid (96%, *v*/*v*). The flow rate was 1.0 mL min^{-1}.

2.6. Determination of Content of Methanol, Ethanol and Acetaldehyde

According to the protocol described by Zou et al. [14] with minor modifications, 15 fruits were randomly selected and divided into three biological replicates (5 per replicate). The pulp was separated from peel and used for juicing. A total of 5 mL of juice was transferred into a 20 mL head-space vial and equilibrated for 15 min at 80 °C in an autosampler (HSS65.5). Subsequently, 1 mL head-space gas was automatically injected into a gas chromatography (Shimadzu GC-2010 Plus) equipped with a flame ionization detector (FID). GC conditions: DB-624 capillary column (60 m × 0.25 mm i.d., 1.4 μm film); oven

temperature, 40 °C; injector temperature, 250 °C; FID temperature, 250 °C; carrier gas, H_2 at a constant pressure of 30 mL min^{-1}; split ratio, 1:10.

2.7. Determination of Volatility Profile in Juice Samples

HS-SPME (head-space solid-phase micro-extraction) procedure

Fifteen fruits were randomly selected and divided into three biological replicates (5 per replicate). The pulp was separated from the peel and used for juicing. Five milliliters of clarified juice, 1.0 g NaCl (analytical grade) and 10 µL internal standard solution (4-methyl-2-pentanol, 1.0018 g L^{-1}) were mixed in a 15 mL amber vial sealed with a PTFE-faced silicone septum. Vials were loaded into a CTC Combi-PAL auto-sampler (CTC Analytics, Zwingen, Switzerland). After being equilibrated for 30 min at 40 °C under magnetic stirring (250 rpm), a 2.0 cm DVB/CAR/PDMS fiber (50/30 µm; Supelco, Bellefonte, PA, USA) was exposed to the head-space for 30 min at the same temperature and stirring rate to adsorb volatile compounds.

GC–MS conditions

Volatile compounds were desorbed in the GC injector (250 °C) for 8 min in non-split mode. Separation was performed on an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film) with helium at 1 mL min^{-1}. The oven temperature was initially set as 50 °C for 1.0 min, and then raised by 3 °C min^{-1} to 220 °C, and held for 5.0 min. Mass spectra were acquired by EI mode (70 eV); the temperature of quadrupole, transfer line and ion source was 150 °C, 280 °C and 250 °C, respectively. Full-scan spectra were recorded from 29 to 350 m/z.

Qualitative and quantitative analysis of volatile compounds

The C_6–C_{24} n-alkanes were analyzed under identical GC-MS conditions to determine the linear retention index (RI) of each compound. Peaks were deconvoluted with an AMDIS (Automated Mass Spectral Deconvolution and Identification System), and their spectra were matched against the NIST14 library (match factor ≥ 800; ΔRI ≤ 10). The RI was calculated as follows:

$$RI = 100\,n + 100 \times (tR_{(X)} - tR_{(n)})/(tR_{(n+1)} - tR_{(n)}), \quad (2)$$

where RI was the retention time of the target component; $tR_{(x)}$, $tR_{(n)}$ and $tR_{(n+1)}$ were the retention times of the component, n-alkanes and (n + 1)-alkanes.

Compound identification was based on the linear retention index (RI) and electron-impact mass spectrum acquired under identical GC-MS conditions. For compounds lacking authentic standards, a semi-identification strategy was adopted: the experimental RI was compared with (i) literature values obtained on an equivalent column and temperature program, and (ii) the NIST14 library. A compound was tentatively assigned only when the absolute difference between experimental and reference RI was ≤50 units and the mass-spectrum match factor (NIST) was ≥800.

For quantification, the characteristic ion (or ion cluster) of each target compound was extracted and integrated. The corresponding ion of the internal standard (4-methyl-2-pentanol, m/z 57) was treated identically. The ratio of the target peak area to the internal standard peak area was used as the quantitative response; results are reported as relative peak-area ratios unless calibrated.

2.8. Statistical Analysis

The variance in data was analyzed using SPSS software package release 18.0 (SPSS Inc.). Multiple comparisons were performed by one-way ANOVA based on *Duncan's* multiple

range tests, while paired-sample *t*-tests were performed to test the statistical significance between two samples at the same time point. The partial least squares discriminant analysis (PLS-DA, for screening of differential accumulated metabolites) and drawing of a correlation network graph was conducted using the OmicStudio tools at https://www.omicstudio.cn/tool(accessed on 2 September 2025) [15].

3. Results

3.1. Changes in Pericarp Color and Flesh Quality During LTS and Effect of LTS Plus 7 Days of RTS on Them

As shown in Figure 1a, during 0 to 120 d of LTS, the *L** value of pericarp gradually decreased, then fluctuated and slightly rebounded from 120 to 180 d of LTS. LTS_0d + RTS_7d, LTS_20d + RTS_7d, LTS_40d + RTS_7d, and LTS_60d + RTS_7d did not result in a significantly higher *L** value, whereas LTS_80d + RTS_7d, LTS_100d + RTS_7d, and LTS_120d + RTS_7d resulted in a significantly higher *L** value. In total, the RTS of 7 days made the 'Orah' mandarin fruit brighter.

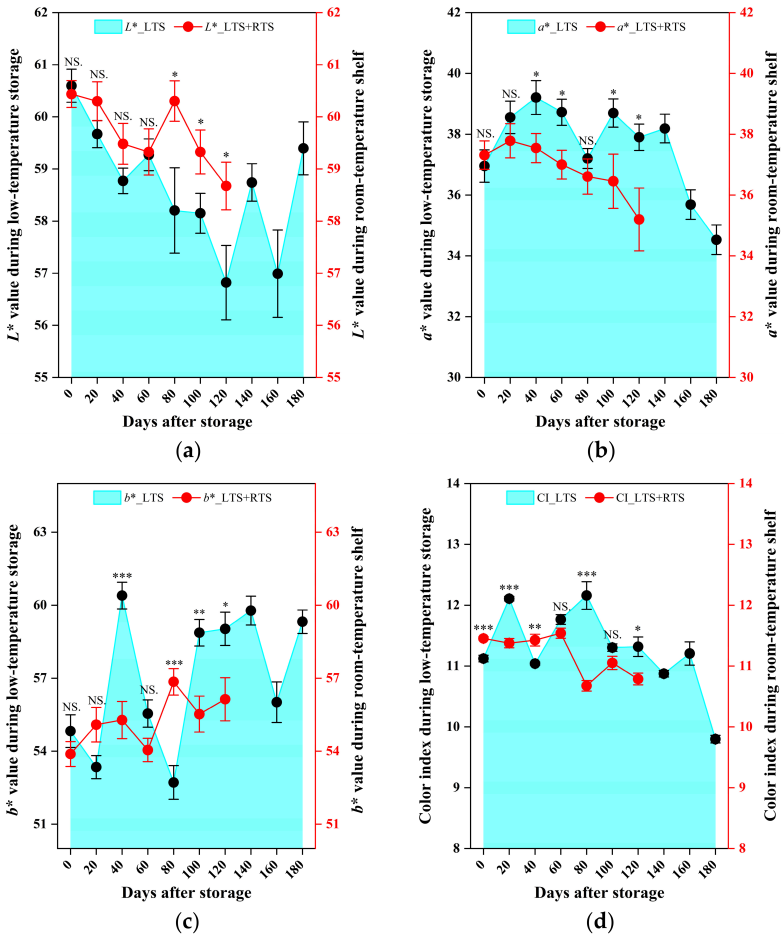


Figure 1. The changes in chromatic values and color index of 'Orah' mandarin and the effect of 7-days-room-temperature shelf on them. (**a**) *L**; (**b**) *a**; (**c**) *b**; (**d**) color index (CI). Asterisks indicate significant difference between the LTS and the LTS + RTS at the same time point: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

As shown in Figure 1b, during the LTS, the *a** value of pericarp gradually increased and reached a peak value at 40 d of LTS, but rapidly decreased from 40 to 80 d of LTS and then fluctuated and significantly decreased from 140 to 180 d of LTS. The RTS of 7 days resulted in a lower *a** value of pericarp at LTS_40d + RTS_7d, LTS_60d + RTS_7d, LTS_100d

+ RTS_7d, and LTS_120d + RTS_7d. In total, the RTS of 7 days reduced the redness of the 'Orah' mandarin.

The *b** value of pericarp gradually increased from 0 to 40 d of LTS and reached a peak value at 40 d, but then rapidly decreased from 40 to 80 d of LTS. The *b** value rapidly rebounded from 80 to 140 d and reached a peak value at 140 d. The RTS of 7 days resulted in a higher *b** value of pericarp at LTS_80d + RTS_7d, but in total, the RTS of 7 days resulted in a lower *b** value of pericarp, especially at LTS_100d + RTS_7d and LTS_120d + RTS_7d (Figure 1c). In total, the RTS of 7 days reduced the yellowness of the 'Orah' mandarin.

A single chromatic value cannot reflect the overall impact of RTS of 7 days on the pericarp appearance of low-temperature stored 'Orah' mandarin (Figure S1). Therefore, we introduced the color index CI as an indicator. As shown in Figure 1d, although LTS_0d + RTS_7d and LTS_40d + RTS_7d resulted in a significantly higher CI of pericarp, in total, the RTS of 7 days resulted in a significantly lower CI value of pericarp, especially at LTS_20d + RTS_7d, LTS_80d + RTS_7d and LTS_120d + RTS_7d.

As shown in Figure 2a, during the earlier stages of LTS, the TSS content in pulp slightly decreased from 0 d (13.70%) to 20 d (13.37%), then rebounded and reached a peak value (14.03%) at 60 d, but then continuously decreased to 12.48% at 180 d of LTS. The RTS of 7 days did not result in lower TSS content in pulp at LTS_0d + RTS_7d, LTS_20d + RTS_7d, and LTS_40d + RTS_7d, but led to significantly lower TSS content at LTS_60d + RTS_7d to LTS_120d + RTS_7d (12.48%).

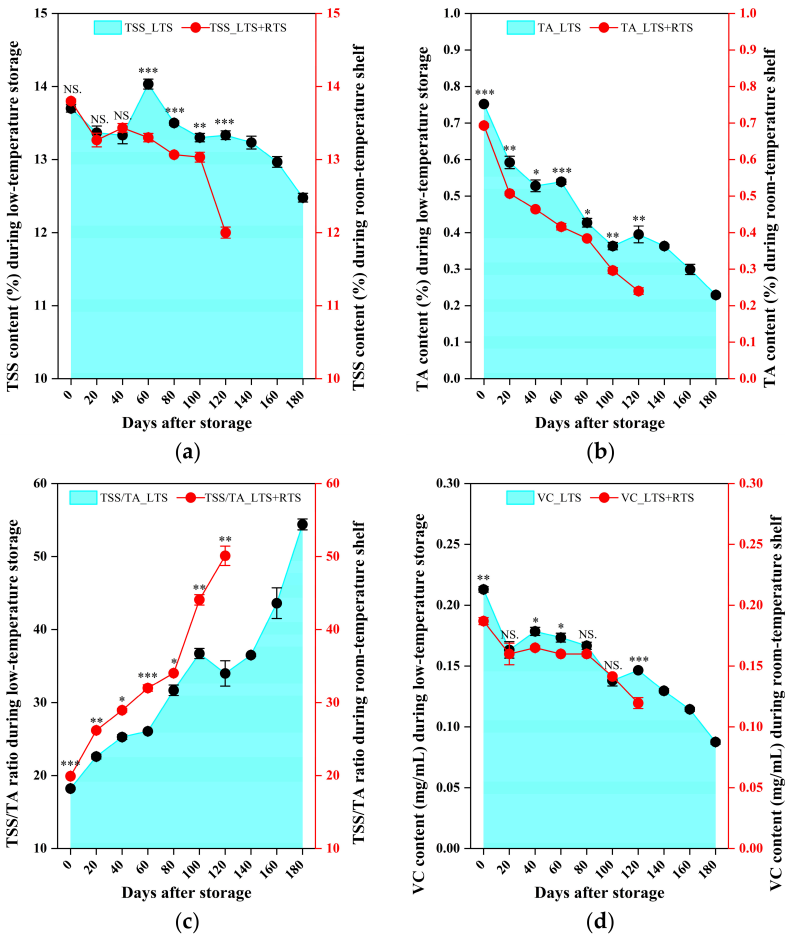


Figure 2. The changes in TSS, TA, TSS/TA and VC in 'Orah' mandarin pulp and the effect of 7-days-room-temperature shelf on them. (**a**) TSS content; (**b**) TA content; (**c**) TSS/T; (**d**) VC content. Asterisks indicate significant difference between the LTS and the LTS + RTS at the same time point: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The TA content in pulp continuously decreased from 0 d of LTS (0.75%) to 180 d of LTS (0.23%). The RTS of 7 days resulted in significantly lower TA content throughout the whole LTS. Among the seven time points of RTS, the LTS_120d + RTS_7d resulted in the maximum reduction in TA (39.00%) (Figure 2b).

The TSS/TA ratio is an important parameter affecting the flavor of citrus pulp. The RTS of 7 days resulted in a significantly higher TSS/TA ratio throughout the whole LTS (Figure 2c). Similar to the TA content, the VC rapidly and continuously decreased throughout the LTS (Figure 2d). The RTS of 7 days resulted in a lower VC content, especially at LTS_0d + RTS_7d, LTS_40d + RTS_7d, LTS_60d + RTS_7d and LTS_120d + RTS_7d. The above results indicated that the RTS of 7 days after room-temperature storage, especially LTS_120d + RTS_7d, significantly altered the intrinsic quality of 'Orah' mandarin, and generally accelerated the consumption of TSS, TA, and VC in the pulp, leading to a deterioration in taste.

3.2. Changes in Content of Sugars and Organic Acids in Pulp During LTS and Effect of LTS Plus 7 Days of RTS on Them

The contents of the three major sugars and two organic acids in the pulp were further analyzed (Figure 3). The results showed that the sucrose content in the pulp rapidly decreased from 124.16 mg/g to 47.22 mg/g during LTS. After an RTS of 7 days, the sucrose content in the pulp at LTS_0d + RTS_7d, LTS_20d + RTS_7d, LTS_40d + RTS_7d, LTS_60d + RTS_7d, LTS_80d + RTS_7d, LTS_100d + RTS_7d, LTS_120d + RTS_7d were all significantly decreased, with the sucrose content at LTS_120d + RTS_7d showing the greatest decrease by 38.15% (Figure 3a).

Along with the rapid decrease in sucrose, the contents of glucose and fructose in the pulp continuously increased from 0 d to 100 d of LTS and then fluctuated in a small range (Figure 3b,c). Interestingly, 7 days of RTS after being stored at LTS produced different effects on the contents of glucose and fructose (Figure 3b,c). LTS_0d + RTS_7d resulted in higher glucose in the pulp, but LTS_60d + RTS_7d, LTS_80d + RTS_7d, LTS_100d + RTS_7d, and LTS_120d + RTS_7d resulted in decreased glucose content in the pulp (Figure 3b), while LTS_0d + RTS_7d, LTS_20d + RTS_7d, LTS_40d + RTS_7d, LTS_60d + RTS_7d, and LTS_80d + RTS_7d resulted in increased glucose in the pulp, but LTS_100d + RTS_7d and LTS_120d + RTS_7d resulted in decreased glucose content in the pulp.

As shown in Figure 3d, in summary, all of the LTS_0d + RTS_7d, LTS_20d + RTS_7d, LTS_40d + RTS_7d, LTS_60d + RTS_7d, LTS_80d + RTS_7d, and LTS_100d + RTS_7d led to a significant decrease in the ratio of sucrose/hexose in the pulp, while the LTS_20d + RTS_7d, LTS_40d + RTS_7d, LTS_60d + RTS_7d, LTS_80d + RTS_7d, LTS_100d + RTS_7d and LTS_120d + RTS_7d caused a significant decrease in the total content of these three sugars in the pulp (Supplementary Figure S3a). These effects from the 7 days of RTS after an LTS, especially LTS_120d + RTS_7d, performed a negative effect on the sweetness and taste of 'Orah' mandarin.

During the 0 to 180 days of LTS, the contents of the two organic acids in the pulp gradually decreased. The content of citric acid dropped from 8.66 mg/g to 5.33 mg/g, while that of malic acid decreased from 4.98 mg/g to 1.88 mg/g. The 7 days of RTS after being refrigerated for 0 days (LTS_0d + RTS_7d), 20 days (LTS_20d + RTS_7d) and 60 days (LTS_60d + RTS_7d) resulted in a significantly increased content of citric acid content in the pulp. However, the 7 days of RTS after being refrigerated for 120 days (LTS_120d + RTS_7d) led to sharply decreased citric acid by 25.69% in the pulp (Figure 3e). All of the treatments with LTS + RTS_7d (LTS_0d + RTS_7d, LTS_20d + RTS_7d, LTS_40d + RTS_7d, LTS_60d + RTS_7d, LTS_80d + RTS_7d, LTS_100d + RTS_7d and LTS_120d + RTS_7d) resulted in decreased content of malic acid in the pulp. Among them, the effects of LTS_60d + RTS_7d and LTS_120d + RTS_7d were significant (Figure 3f). The total content of these two organic

acids in the pulp was significantly changed by the treatments with LTS + RTS_7d, which is significantly increased in the LTS_20d + RTS_7d group, while significantly decreased in the LTS_120d + RTS_7d group (Supplementary Figure S3b).

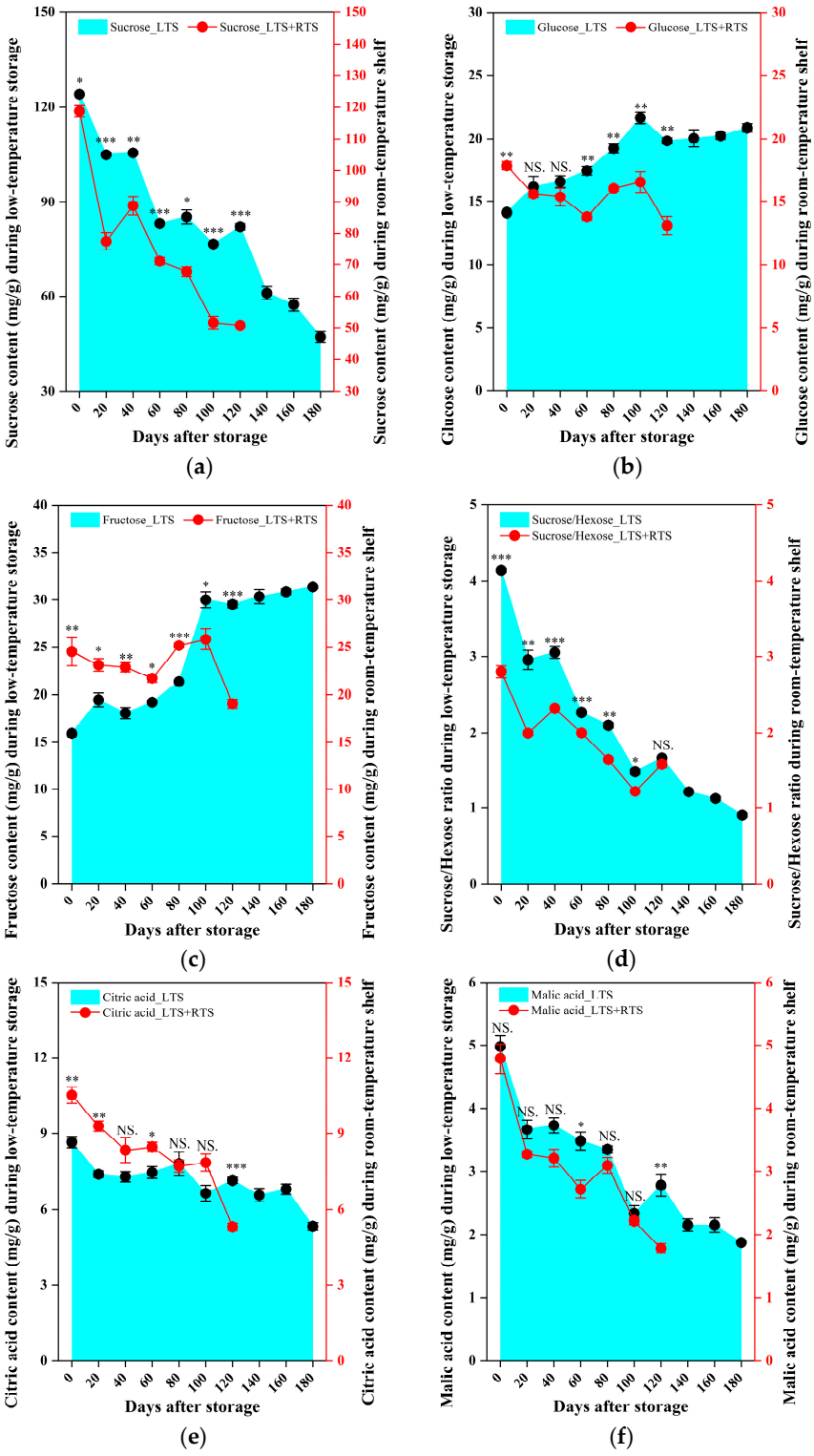


Figure 3. The changes in sucrose, glucose, fructose, sucrose/hexose, citric acid and malic acid in 'Orah' mandarin pulp and the effect of 7-days-room-temperature shelf on them. (**a**) Sucrose content; (**b**) glucose content; (**c**) fructose content; (**d**) sucrose/hexose; (**e**) citric acid content; (**f**) malic acid content. Asterisks indicate significant difference between the LTS and the LTS + RTS at the same time point: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.3. Changes in Content of Methanol, Ethanol, and Acetaldehyde in Pulp During LTS and Effect of LTS + 7 d RTS on Them

During the period from 0 to 180 days of LTS, the content of ethanol was the highest among the three off-flavor substances in the 'Orah' mandarin pulp, followed by methanol and acetaldehyde. Their content increased continuously along with the LTS (Figure 4). During the LTS, the ethanol content decreased from 61.62 mg/L at 0 d to 45.47 mg/L at 40 d, but then rose sharply (132.59 mg/L at 60 d; 133.31 mg/L at 80 d), reached 349.84 mg/L at 100 d and 395.20 mg/L at 120 d, and finally increased to 635.34 mg/L at 180 d (Figure 4a). All of the treatments with 7 days of RTS after being stored at low-temperature for 0 d, 20 d, 40 d, 60 d, 80 d, 100 d and 120 d resulted in a significantly higher accumulation of ethanol. The ethanol content of the pulp sample from LTS_100d + RTS_7d was 2153.15 mg/L, which was 13.06 times that of the pulp sample from LTS_100d, while the ethanol content of the pulp sample from LTS_120d + RTS_7d was 4222.50 mg/L, which was 10.69 times that of the pulp sample from LTS_120d.

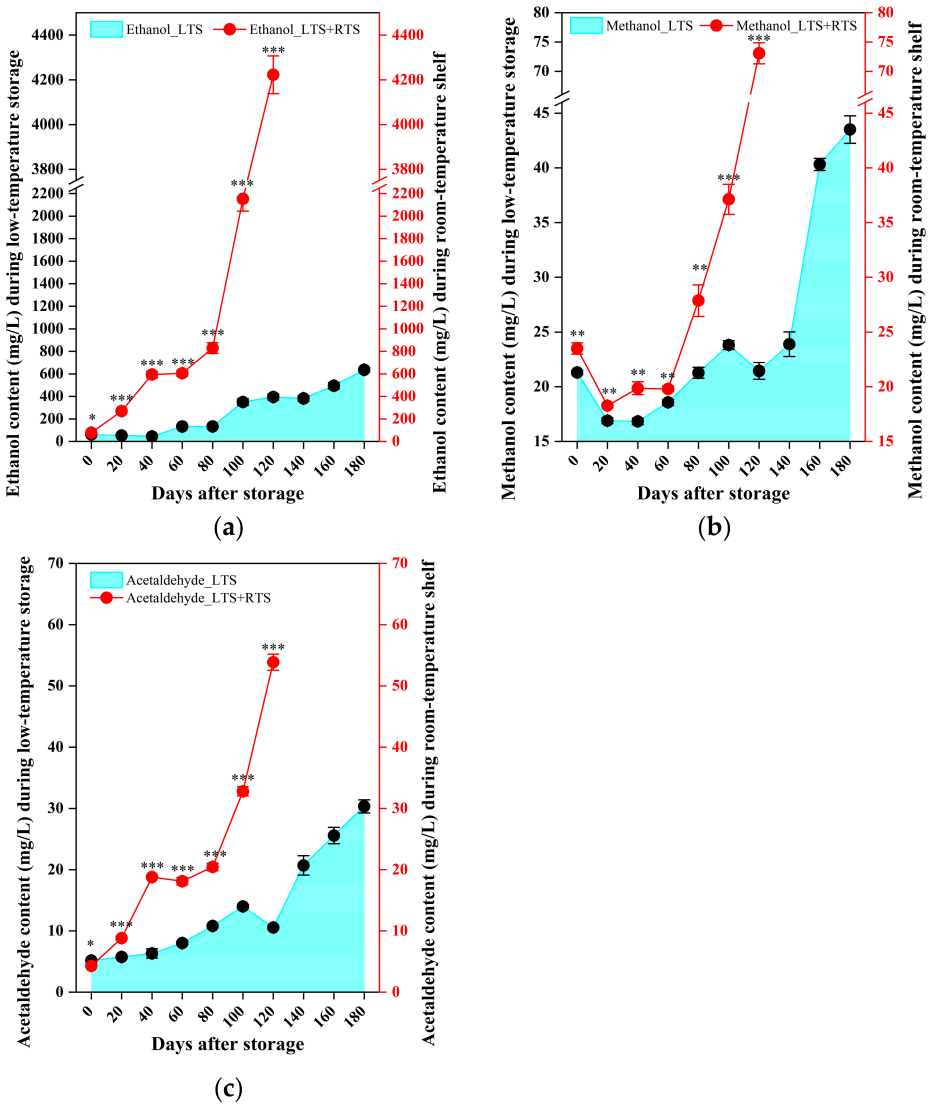


Figure 4. The changes in methanol, ethanol, and acetaldehyde content in 'Orah' mandarin pulp and the effect of 7-days-room-temperature shelf on them. (**a**) Ethanol content; (**b**) methanol content; (**c**) acetaldehyde content. Asterisks indicate significant difference between the LTS and the LTS + RTS at the same time point: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Similarly, the methanol content in pulp decreased slightly from 21.30 mg/L at 0 d to 18.57 mg/L at 60 d, but increased to 23.80 mg/L at 100 d, and then fluctuated. After that, it rapidly rose to 43.50 mg/L at 180 d (Figure 4b). Interestingly, the treatments with LTS_0d + RTS_7d, LTS_20d + RTS_7d, LTS_40d + RTS_7d, and LTS_60d + RTS_7d just resulted in a slight increase in the methanol content in pulp, while the treatments with 7 days of RTS after being stored at low-temperature for 80 d, 100 d, and 120 d resulted in 1.31 times that of LTS_80d, 1.56 times that of LTS_100d, and 3.41 times that of LTS_120d. The overall increase in methanol content in pulp was not as significant as that of ethanol.

The acetaldehyde content in pulp gradually increased from 5.15 mg/L at 0 d to 13.99 mg/L at 100 d, slightly decreased to 10.55 mg/L at 120 d, but then rapidly rose to 30.34 mg/L at 180 d (Figure 4c). The LTS_40d + RTS_7d, LTS_60d + RTS_7d, LTS_80d + RTS_7d, LTS_100d + RTS_7d, and LTS_120d + RTS_7d resulted in 2.97 times that of LTS_40d, 2.26 times that of LTS_60d, 1.89 times that of LTS_80d, 2.34 times that of LTS_100d, and 5.10 times of that of LTS_120d.

Overall, the content of all the three off-flavor compounds was significantly up-regulated when subjected to a RTS after the LTS of 0 d, 20 d, 40 d, 60 d, 80 d, 100 d, 120 d and 180 d. Among all the periods, the up-regulation at LTS_120d + RTS_7d was the greatest: the contents of ethanol, methanol and acetaldehyde increased by 10.69 times (from 395.20 mg/L to 4222.50 mg/L), 3.41 times (from 21.45 mg/L to 73.07 mg/L) and 5.10 times (from 10.56 mg/L to 53.87 mg/L), respectively (Figure 4). Among the three off-flavor substances, ethanol showed the largest increase, indicating that the RTS significantly accelerated the production of ethanol.

3.4. Changes in Content of Volatile Compounds in Pulp During LTS and Effect of LTS + 7 d RTS on Them

To systematically study the variation patterns of volatile compounds during the LTS, samples at 0 d (LTS_0d), 60 d (LTS_60d), 120 d (LTS_120d), and 180 d (LTS_180d) of LTS and the 7 days' RTS after the LTS_120d (LTS_120d + RTS_7d) were collected for GC-MS/MS analysis. As shown in Figure 5a,b, a total of 43 volatile compounds were detected, including 21 lipids (glyceroyl-) (48.84%), 13 terpenoids (30.23%), 4 organic acids (9.30%), 2 tetrahydropyrans (4.65%), 1 alcohol (2.33%), 1 aldehyde (2.33%), and 1 ester (2.33%). Specifically, the lipids (glyceroyl-) were composed of 11 fatty alcohols, 5 fatty aldehydes, 4 fatty esters, and 1 oxygenated hydrocarbon. Among the 43 detected compounds, 33 were annotated in the KEGG database with compound IDs.

The PCA component analysis revealed that the three biological replicates of LTS_0d were distributed in the upper right quarter of the quadrant, the samples of LTS_60d and LTS_120d were clustered in the lower-half of PCA2, namely the negative axis part, while the samples of LTS_120d + RTS_7d and LTS_180d were distributed in the upper left quarter, but these two groups of samples were significantly separated (Figure 5c). As shown in the loading plot of volatile metabolites, all of the 13 terpenoids were distributed in the upper right quarter of the quadrant, while all of the 4 organic acids were distributed in the upper left quarter of the quadrant, indicating that these two classes of volatile compounds significantly contributed to the differences between the sample groups (Figure 5d).

The results from the cluster heatmap of volatile compounds were consistent with the results of the PCA (Figure 5c,e). The three biological replicates of each sample were clustered together, indicating good repeatability. The samples of the LTS_0d group were separated from those of other groups, while the samples at LTS_60d and LTS_120d were clustered in one group, and the samples at LTS_120d + RTS_7d and LTS_180d were clustered together (Figure 5e).

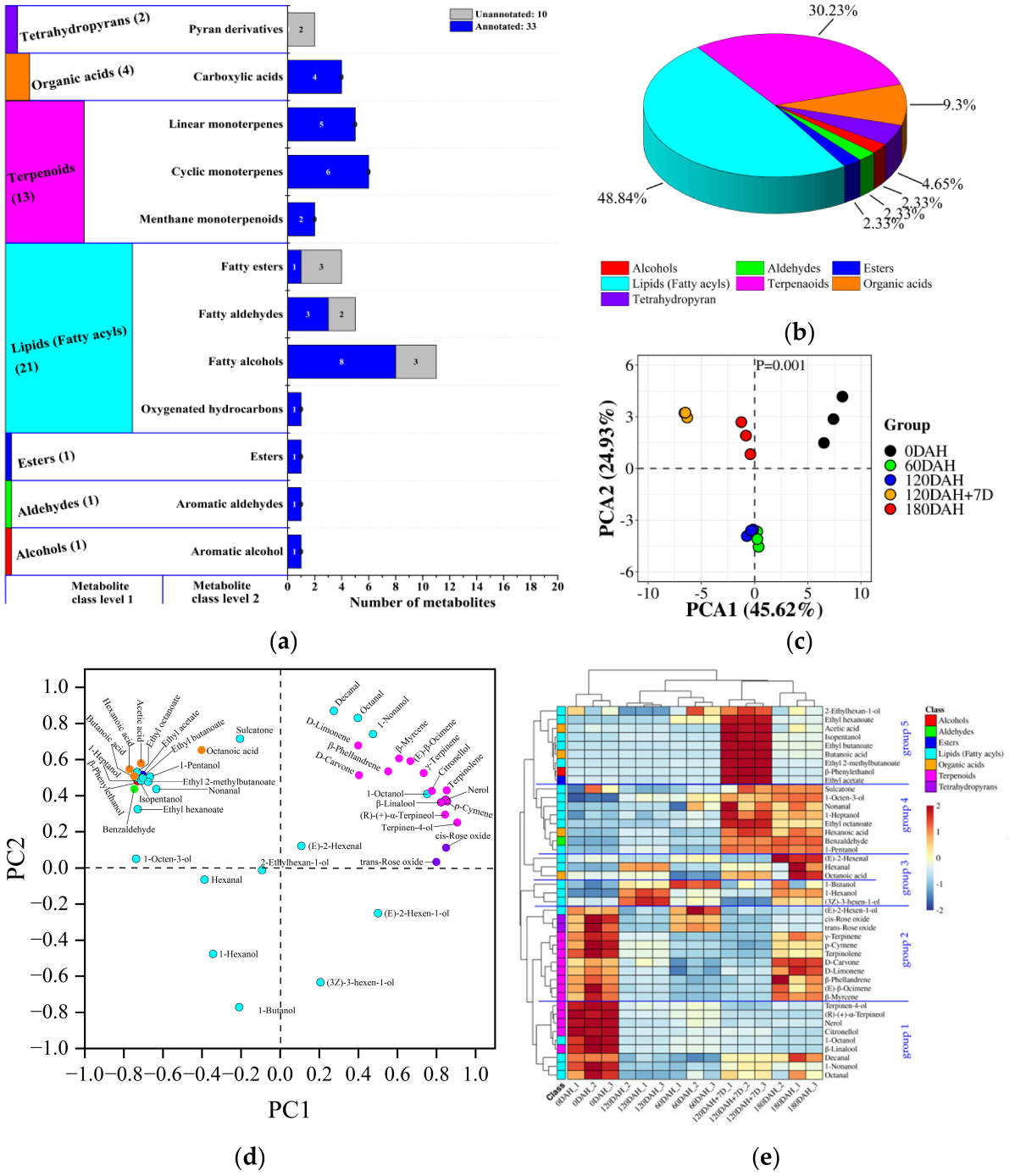


Figure 5. Statistics of the volatile metabolites in the 'Orah' mandarin juice from 0 DAH, 60 DAH, 120 DAH, 180 DAH and 7-days-room-temperature shelf after 120-days LTS. (**a**) Statistics of the detected and annotated metabolites; (**b**) percentage of the metabolites in each class; (**c**) PCA analysis of 43 detected volatile metabolites; (**d**) loading of metabolites in the PC1 and PC2 plot; (**e**) cluster heatmap analysis of metabolites in each class. 0DAH, LTS_0d; 60DAH, LTS_60d; 120DAH, LTS_120d; 180DAH, LTS_180d; 120DAH + 7D, LTS_120d + RTS_7d.

In the heatmap, the volatile compounds could be classified into five groups based on their variation patterns: group 1: the content of four lipids and five terpenoids showed the highest level at the beginning of storage (LTS_0d), then dropped rapidly at 60DAH or the later stages and remained at a low level; group 2: their content showed the highest level at the beginning of storage (LTS_0d), then dropped rapidly, but returned to be higher at LTS_60d (one lipid and two tetrahydropyrans) or LTS_180d (eight terpenoids); group 3: the

content of five lipids and one organic acid was low at the beginning of storage (LTS_0d), and reached a peak at LTS_60d, LTS_120d or LTS_180d; group 4: the content of six lipids, one organic acid and one aldehyde was low at the beginning of storage (LTS_0d), then fluctuated slightly or remained at a low level during the subsequent period, but then reached the highest value at both of LTS_120d + RTS_7d and LTS_180d; group 5 (including five lipids, two organic acids, one alcohol and one ester): their abundance did not change significantly throughout the LTS, but increased significantly after being treated with LTS_120d + RTS_7d (Figure 5e).

It was interesting to note that all of terpenoids were clustered into group 1 and group 2. Among them, five terpenoids (terpinen-4-ol, (R)-(+)-α-terpineol, nerol, citronellol, 1-octanol, β-linalool) showed the highest level at the beginning of storage (LTS_0d), but rapidly dropped to an extremely low level, while the other eight terpenoids (γ-terpinene, *p*-cymene, terpinolene, D-carvone, D-limonene, β-phellandrene, (E)-β-ocimene, β-myrcene) showed the highest level at LTS_0d, then rapidly dropped, but returned to be higher at LTS_180d. This result suggested that the terpenoids in 'Orah' mandarin juice were prone to loss during the low-temperature storage. Also, it was worth noting that nine volatile compounds composed of five lipids (2-ethylhexan-1-ol, ethyl hexanoate, isopentanol, ethyl butanoate, ethyl 2-methylbutanoate), two organic acids (acetic acid, butanoic acid), one alcohol (β-phenylethanol) and one ester (ethyl acetate) were specially up-regulated in the samples of LTS_120d + RTS_7d (Figure 5e).

3.5. Significantly Differently Accumulated Metabolites During LTS and LTS + RTS

We conducted a PLS-DA using the contents of vitamin C, sucrose, glucose, fructose, citric acid, malic acid, methanol, ethanol, acetaldehyde and 43 above-mentioned volatile substances to obtain variable importance in projection (VIP) values of each compound and screen the differential accumulated metabolites with VIP value ≥ 1, p-value < 0.05 and $|\log_2(FC)| \geq 1$ (Figure 6).

Compared with the LTS_0d group, four metabolites, which were all lipids (four fatty esters: ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate) were up-regulated, while eight metabolites including seven terpenoids (β-phellandrene, (R)-(+)-α-terpineol, citronellol, (E)-β-ocimene, β-myrcene, β-linalool, terpinolene) and one lipid (1-octanol) were down-regulated in the LTS_60d group (Figure 6a). Compared with the LTS_0d group, 9 metabolites including 7 lipids (ethyl hexanoate, ethyl butanoate, hexanal, 1-hexanol, 1-pentanol, (3Z)-3-hexen-1-ol, isopentanol), 1 alcohol (ethanol), and 1 organic acid (hexanoic acid) were up-regulated, while 14 metabolites including 8 terpenoids ((R)-(+)-α-terpineol, citronellol, (E)-β-ocimene, nerol, β-myrcene, β-linalool, terpinen-4-ol, terpinolene), 3 lipids (1-nonanol, 1-octanol, octanal), 2 tetrahydropyran (cis-rose oxide, trans-rose oxide) and 1 ester (ethyl acetate) were down-regulated in the LTS_120d group (Figure 6b).

Compared with the LTS_0d, 10 metabolites including six lipids (two fatty alcohols: 1-pentanol, isopentanol; four fatty esters: ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate), two aldehydes (benzaldehyde, acetaldehyde), one organic acid (hexanoic acid), and one alcohol (ethanol) were up-regulated, but four metabolites including three terpenoids ((R)-(+)-α-terpineol, citronellol, β-linalool) and one lipid (1-octanol) were down-regulated in the LTS_180d group (Figure 6c). The Venn analysis indicated the continuous and steady decrease in the content of 1-octanol, (R)-(+)-α-terpineol, citronellol and β-linalool, which might be important indicators of the loss of aroma in the 'Orah' mandarin juice (Figure S5a).

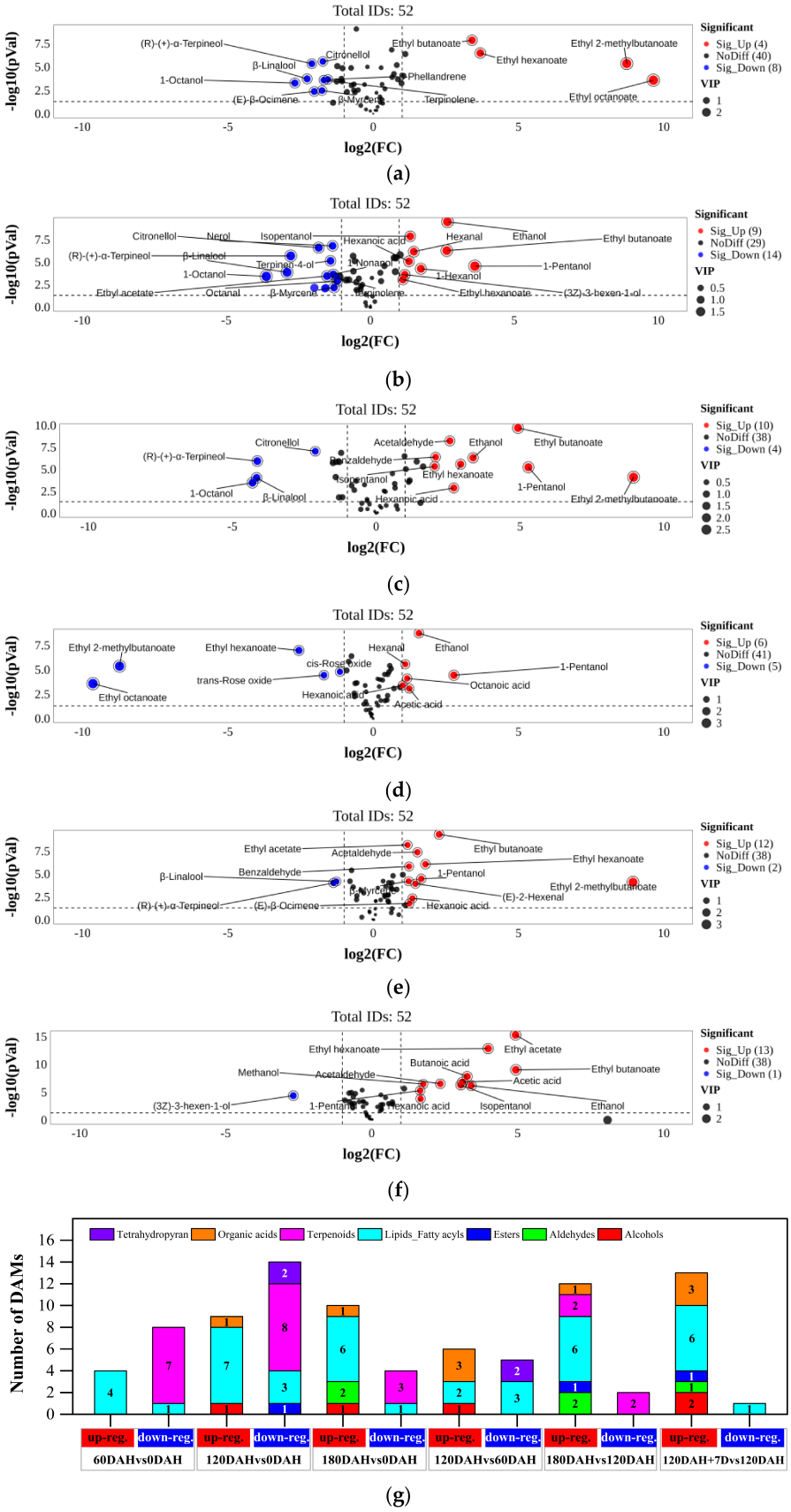


Figure 6. Volcano plot and DAMs screening based on the PLS-DA of 52 detected metabolites in the 'Orah' mandarin juice in this work. DAMs: VIP > 1, fold change > 2, *p*-value < 0.05) at different stages. (**a**) LTS_60d vs. LTS_0d; (**b**) LTS_120d vs. LTS_0d; (**c**) LTS_180d vs. LTS_0d; (**d**) LTS_120d vs. LTS_60dH; (**e**) LTS_180d vs. LTS_120d; (**f**) LTS_120d + RTS_7d vs. LTS_120d; (**g**) number of DAMs.

From the perspective of phased changes in metabolites, compared with the LTS_60d group, six metabolites including three organic acids (octanoic acid, hexanoic acid, acetic acid), two lipids (fatty alcohols: 1-pentanol, hexanal) and one alcohol (ethanol) were up-regulated, while five metabolites including three lipids (fatty esters: ethyl 2-methylbutanoate, ethyl hexanoate, ethyl octanoate) and two tetrahydropyrans (cis-rose oxide, trans-rose oxide) were down-regulated in the LTS_120d group (Figure 6d). Compared with the LTS_120d group, 12 metabolites including 6 lipids (fatty alcohols: 1-pentanol; fatty aldehydes: (E)-2-hexenal; fatty esters: ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate), 2 terpenoids ((E)-β-ocimene, β-myrcene), 2 acetaldehydes (benzaldehyde, acetaldehyde), 1 organic acid (hexanoic acid) and 1 ester (ethyl acetate) were up-regulated, but 2 terpenoids ((R)-(+)-α-terpineol, β-linalool) were down-regulated in the LTS_180d group (Figure 6e). These results indicated that content of volatile compounds underwent a significant change from LTS_0d to LTS_180d.

Compared with the LTS_120d group, 13 metabolites including 6 lipids (fatty alcohols: 1-pentanol, isopentanol; fatty esters: ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate), 3 organic acids (hexanoic acid, butanoic acid, acetic acid), 2 alcohols (methanol, ethanol), 1 aldehyde (acetaldehyde) and 1 ester (ethyl acetate) were up-regulated, while only 1 lipid (fatty alcohols: (3Z)-3-hexen-1-ol) was down-regulated in the LTS_120d + 7d group.

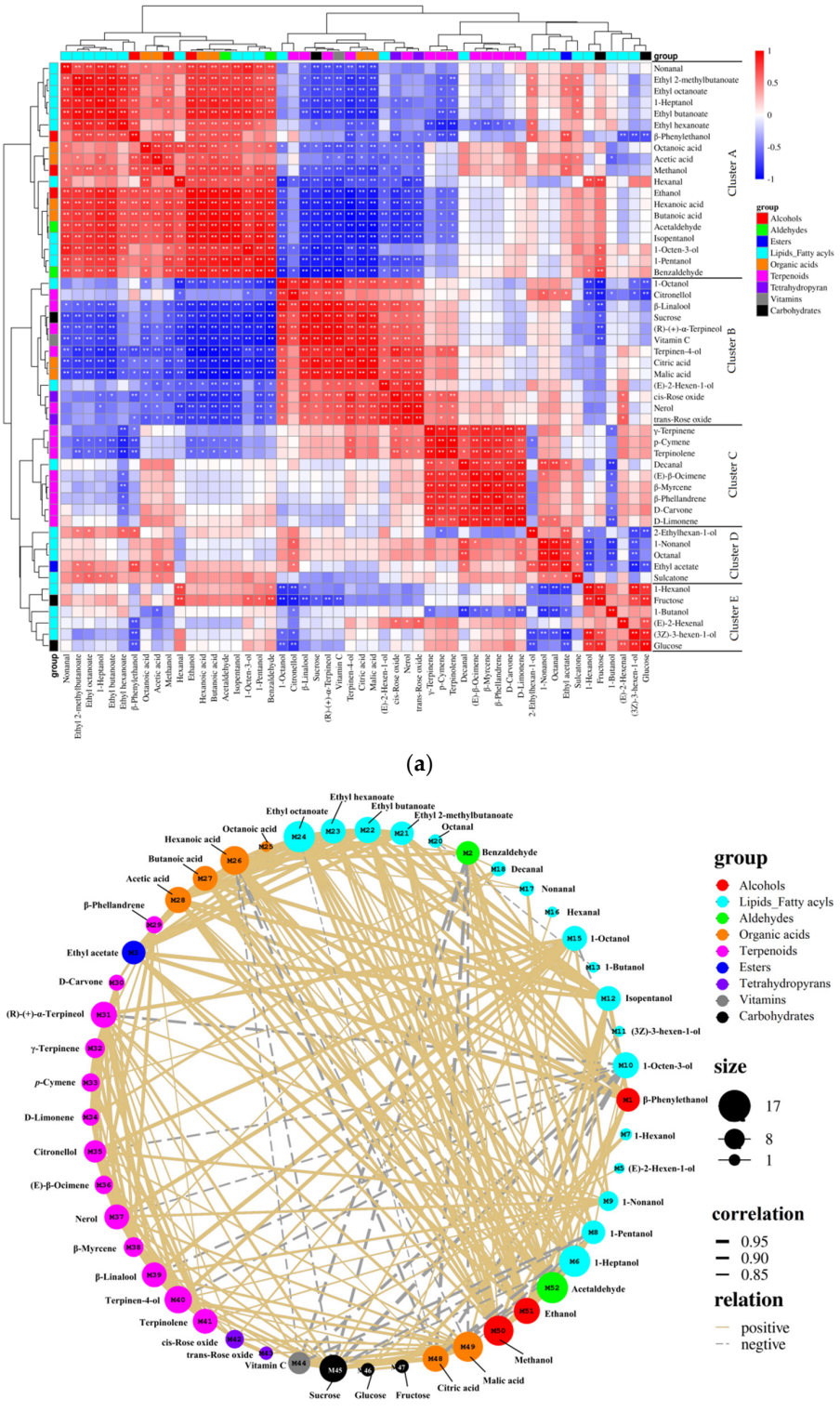
The Venn analysis indicated that four lipids (fatty esters: ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate) were commonly up-regulated at the first (LTS_0d to LTS_60d) and last stage (LTS_120d to LTS_180d), which were also induced by the 7 days of RTS at LTS_120d (namely LTS_120d + 7d); 1-pentanol and hexanoic acid were commonly up-regulated at the last two stages (LTS_60d to LTS_120d and LTS_120d to LTS_180d), which were also up-regulated in the LTS_120d + 7d group; ethyl acetate and acetaldehyde were up-regulated at the later stages of LTS (namely, LTS_120d to LTS_180d) and also induced by the 7 days of RTS at LTS_120d (Figure S5b). The above results indicated that the 7 days of RTS at LTS_120d produced similar effects as LTS_180d. The 7 days of RTS up-regulated the accumulation of ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate (fatty esters), ethyl acetate (ester), acetaldehyde (aldehyde), 1-pentanol (fatty alcohols) and hexanoic acid (organic acid) without exacerbating the decline of terpenoids, which experienced a significant decrease within the first 60 days of LTS.

3.6. Significant Correlation Between the Detected Metabolites During LTS and LTS + RTS

In order to screen out the potential associations among metabolites, correlation analysis was performed using the Pearson correlation coefficient to screen the significant pairwise correlation ($R > 0.80$ and significance > 0.05) (Figure 7). As shown in Figure 7a, a correlation-cluster heatmap using the 52 detected metabolites was constructed in this study. These metabolites were clustered into five clusters, within which the members showed high positive correlation with each other. The biggest group (cluster A) included 19 metabolites containing 10 lipids (1-heptanol, 1-pentanol, 1-octen-3-ol, isopentanol, hexanal, nonanal, ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate), 4 organic acids (octanoic acid, hexanoic acid, butanoic acid, acetic acid), 3 alcohols (ethanol, methanol, β-phenylethanol), and 2 aldehydes (benzaldehyde, acetaldehyde).

The second biggest group (cluster B) included 13 metabolites, which mostly showed negative correlation with the 19 metabolites from cluster A (mainly lipids). Cluster B contained five terpenoids (citronellol, β-linalool, (R)-(+)-α-terpineol, terpinen-4-ol, nerol), two lipids (1-octanol, (E)-2-hexen-1-ol), two tetrahydropyrans (cis-rose oxide, trans-rose oxide), two organic acids (citric acid, malic acid), vitamin C, and carbohydrate (sucrose). The third biggest group (cluster C) included nine metabolites, which were mostly terpenoids

(eight terpenoids, only one lipid). Cluster D included four lipids and one ester, while cluster E included four lipids and two carbohydrates (Figure 7a).



(**a**)

(**b**)

Figure 7. Heatmap and network using correlation between the metabolites. The size of node represents the number of correlation pairs. (**a**) Heatmap of the correlation between 52 detected metabolites; (**b**) correlation network of paired metabolites with correlation > 0.8. *p*-value of the correlation coefficient: *, $p < 0.05$; **, $p < 0.01$.

As shown in Figure 7b, among the alcohols, β-phenylethanol (M1) showed the highest positive correlation (r = 0.9984) with ethyl acetate, while methanol (M50, r = 0.9923) and ethanol (M51, r = 0.9254) showed the highest positive correlation with acetaldehyde. Among the lipids, 1-Octen-3-ol (M10) showed high negative correlation with five terpenoids (M31, M35, M37, M39, M40), two organic acids (M48, M49), vitamin C (M44) and sucrose (M45, r = −0.9804).

4. Discussion

4.1. The Aroma Collapse Preceded the Off-Flavor Outbreak

Volatile organic compounds (VOCs) are the fundamental substances that constitute the flavor chemistry of fruits. In citrus fruit, the main volatile substances are terpenoids, which are the key substances in shaping the characteristic aroma of citrus fruits, due to their diverse structures, strong aroma activity, and extremely low olfactory threshold [16,17]. However, once the fruit was harvested and deprived of the nutrient supply from the parent body, the dynamic balance between the synthesis and degradation of its volatile metabolites irreversibly shifted, ultimately leading to the deterioration of flavor and quality. This study analyzed the physiological characteristic changes of 'Orah' mandarin during the low-temperature storage and revealed the temporal pattern of its aroma quality decline: the terpenoids showed a cliff-like decline in the first 60 days of storage, while the significant accumulation of fermentative odor substances (ethanol, methanol, acetaldehyde) was delayed to 100 days of storage and then burst (Figures 4 and 5e). The temporal shift in these volatile metabolites indicated that, to prevent the significant loss of aroma, LTS should not exceed 60 days, while to avoid the high-level accumulation of off-flavor substances, LTS should not exceed 100 days.

The results of the PCA loading diagram (Figure 5d) and PLS-DA screening (Figure 6) indicated that the 1-octanol, (R)-(+)-α-terpineol, citronellol and β-linalool acted as key indicators of the loss of aroma, whose contents continuously declined during storage in 'Orah' mandarin. It was worth noting that the content of β-linalool, citronellol and (R)-(+)-α-terpineol respectively decreased by 91.33%, 87.27% and 90.21% during the first 60 days of low-temperature storage, resulting in well-known "aroma collapse" phenotype. This phenomenon from our results was consistent with the reports from Tietel et al. in W. Murcott (another tangerine) [18], Farneti et al. in tomato fruit [19], and Butkeviciute et al. (2021) in apple [20], that is, the content of terpenoids in post-harvest fruits shows a downward trend during storage. It was speculated that post-harvest fruit is separated from the mother plant; although the supply of water and nutrient was cut off, it maintains active metabolic activities [21]. Meanwhile, most of the terpenoids are rich in highly reactive sites such as unsaturated double bonds, allyl active hydrogen and exocyclic methylene in their molecular structures, which makes them extremely prone to oxidative degradation in post-harvest reactive oxygen species (ROS) enrichment environments [22]. For example, limonene is attacked by free radicals under heat stress or photo-oxidation to generate monoterpenes or oxidized monoterpenes [23]. Isoprene (such as β-ionone) can also undergo chain oxidation due to excessive exposure to oxygen [24]. Therefore, the oxidative degradation may be an important factor leading to the early collapse of aroma.

The results from other previous works indicated that the effect of LTS temperature on loss of aroma is dependent on species or varieties. For instance, low-temperature storage at 0 °C preserved the β-linalool content in Sunrose grapes, while 10 °C led to the loss of flavor substances [25], but low-temperature dehydration treatment at 8 °C [26] and pre-cooling at 10 °C [27] significantly promoted the accumulation of free and glycoside-bound terpenes in wine grapes and enhanced the flavor of wine. Similarly, storage at 12 °C promoted the biosynthesis of characteristic aroma substances (sesquiterpenes, esters) and inhibited

the synthesis of monoterpenes in grapefruit, while storage at 2 °C triggered a cold stress response, leading to the massive release of monoterpenes (limonene, β-pinene, etc.) [28]. Tietel et al. [29] demonstrated that storage temperature showed no significant effect on the content of terpenoids in cold-tolerant varieties (such as 'Or' citrus), but showed a significant effect on cold-sensitive 'Odem' citrus: storage at 2 °C led to the accumulation of terpenoids such as β-limonene and α-terpinene and their derivatives, causing a strong resinous and musty smell as well as the deterioration of flavor at the later stages of storage. The previous experiments of this study compared the differences in quality changes of 'Orah' mandarin stored at 2–4 °C, and 6–8 °C, and we found that 6–8 °C was the suitable storage temperature [30]. In this study, no sharp loss of total terpenoids but selective loss of specific monoterpene alcohols (linalool, citronellol, α-terpineol) was observed in 'Orah' mandarin during the initial storage at 6–8 °C. It may be attributed to two factors: the key enzymes in the pathway related to terpenoids synthesis (such as terpenes synthase TPS) showed varied patterned cold response among different citrus varieties; secondly, as a loose-skin citrus, 'Orah' mandarin showed differences in membrane lipid unsaturation from other citrus fruits (such as pomelo), and its tolerance to reactive oxygen species (ROS) is also different. Therefore, it is necessary to compare the aroma volatiles of 'Orah' mandarin samples after a low-temperature storage with different temperatures and room-temperature shelves in the future work. Based on the volatile metabolite profiles, it is expected to define the optimal minimum safe temperature and storage period that can not only delay the degradation of flavor substances such as terpenoids, but also avoid cold damage to the 'Orah' mandarin.

In sharp contrast to the cliff-like decline of terpenoids in the early stage of storage, the accumulation of typical metabolites from fermentation (namely ethanol, acetaldehyde and methanol, etc.) in the early stage of storage is relatively slow. Their significant accumulation was not manifested until 100 days after storage (Figure 4). During the early and middle stages of low-temperature storage (0 d to 100 d), the contents of these three fermentation metabolites increased slowly and remained at a relatively low level, which might mainly be attributed to the significant inhibition of the overall metabolic rate of the fruit by low temperature. At this stage, the transcriptional activity and protein abundance of pyruvate decarboxylase and alcohol dehydrogenase were active and at a stable level, and the function of the mitochondrial respiratory chain remained intact, which enables pyruvate to mainly undergo aerobic oxidation through the tricarboxylic acid cycle rather than be metabolized through the anaerobic fermentation pathway [31], and thus the generation and accumulation of ethanol and acetaldehyde was effectively limited. It was worth noting that, although the film thickness of packing bags used in this study was only 0.5 μm and the gas permeability was relatively high, a low-oxygen micro-environment might gradually form inside the bags due to the continuously consuming of O_2 and accumulation of CO_2 during long-term LTS, which might induce anaerobic fermentation in the later stage of storage. The gas composition inside the bag during LTS needs to be monitored in a future study to verify their effects on the fermentation of the 'Orah' mandarin.

The 'aroma collapse'–'off-flavor' cascade in 'Orah' mandarin revealed that the loss of terpenoids might be served as an early-warning indicator of flavor quality deterioration, while the accumulation of off-flavor substances was a landmark event marking the final stage of deterioration. Compared with the traditional passive post-event determination paradigm based on odor threshold detection, predictive early warning of the risk of 'aroma collapse' can be achieved by monitoring the decay rate of characteristic terpenes components (β-linalool, citronellol and α-terpineol). This discovery laid a theoretical foundation for the construction of a precise post-harvest quality monitoring system based on volatile substances. The process of quality deterioration can be effectively blocked before the gener-

ation of odor substances by dynamically monitoring the depletion rate of terpenoids and promptly implementing targeted intervention measures (such as 1-methylcyclopropene treatment, modified atmosphere packaging, etc.) before the critical control window period (storage for 60 days), thereby avoiding irreversible depreciation of commodity value.

4.2. The Safety-Critical Point for Low-Temperature Storage of 'Orah' Mandarin and the Metabolic Basis for Flavor Deterioration on Room-Temperature Shelf After Refrigeration

This study systematically tracked the dynamic changes in the content of methanol, ethanol, acetaldehyde and volatile substances of 'Orah' mandarin during low-temperature storage and subsequent room-temperature shelf. The results showed that the contents of methanol, ethanol and acetaldehyde in the 'Orah' mandarin pulp significantly increased in the later stage of low-temperature storage (120 days to 180 days), but their content did not reach the threshold of sensory odor (2000 mg/L) [3]. The ethanol content in the sample of LTS_100 d + RTS_7d rose sharply from 349.84 mg/L to 2153.15 mg/L (compared LTS_100 d), while that in the sample of LTS_120 d + RTS_7d rose by greater folds (rising from 395.20 mg/L to 4222.50 mg/L) (compared to LTS_120 d), resulting in a distinct off-flavor (Figure 4). The characteristic of ethanol, methanol, and acetaldehyde, that their accumulation was low during low-temperature storage and rapidly increased after being transferred to a room-temperature shelf, might be the metabolic basis for the phenomenon 'off-flavor after being taken out of refrigeration' in citrus production.

Farneti et al. (2015) [19] reported that, after low-temperature storage, the temperature rise would induce the explosion of alcohols in climacteric fruit tomato. A similar phenomenon was also observed in the non-climacteric fruit 'Orah' mandarin in this study, suggesting that anaerobic fermentation induced by temperature rise after low-temperature storage might be a conservative stress response of the fruit and might not be related to ethylene-dependent maturation regulation. Unlike tomatoes, 'Orah' mandarin lacks the typical respiratory peak and ethylene burst. It has been reported that the transformation of fermentation metabolism in citrus is more likely to result from the physical phase transition of the mitochondrial membrane rather than the metabolic reprogramming regulated by ethylene signals. Long-term low-temperature storage led to phase transition and reduced the fluidity of cell membranes, which in turn caused the collapse of mitochondrial membrane potential and dysfunction of the electron transport chain in fruits, ultimately resulting in a cellular energy crisis (litchi fruit) [32]. However, after being taken out of storage, the sudden increase in temperature led to a sharp rise in the respiration rate, caused an explosive accumulation of reactive oxygen species (ROS) and triggered membrane lipid peroxidation reactions (apple fruit) [10]. In addition, the rising respiration accelerated the consumption of oxygen and the accumulation of carbon dioxide within the packaging bag, resulting in hypoxic stress. The above-mentioned superimposed stress leads to the failure of the antioxidant system and the aerobic respiratory system; the metabolic flow of fruit is forced to shift to the anaerobic fermentation pathway to maintain the basic energy supply, resulting in a large accumulation of fermentation products such as ethanol and acetaldehyde. The increase in acetaldehyde was lower than that of ethanol in 'Orah' mandarin during the LTS_120 d + RTS_7d. This might be explained as a direct precursor to ethanol synthesis; acetaldehyde is partially reduced to ethanol by alcohol dehydrogenase (ADH) or oxidized to acetic acid by aldehyde dehydrogenase (ALDH). This speculation was confirmed by the data that the content of acetic acid in the sample of LTS_120 d + RTS_7d was 11.26 folds higher than that in the sample of LTS_120 d (Figure 5e). Methanol, as a key product of plant cell wall metabolism, is mainly produced by the demethylation reaction of homologous galacturonic acid glycan (HG) catalyzed by pectin methylesterase (PME), and its accumulation is usually accompanied by the softening, ripening and senescence processes of fruits [33]. In this study, the methanol content of 'Orah' mandarin

increased sharply during the LTS_120 d + RTS_7d (Figure 4), indicating that the cell wall metabolism and senescence are accelerated. For 'Orah' mandarin, the dynamic monitoring of the O_2/CO_2 concentration inside the bag combined with comparative tests of packaging with different air permeability were necessary to be conducted in our future study to quantitatively distinguish the relative contributions of the warming effect and the gas environmental effect inside the bag on the 'off-flavor after being taken out of refrigeration'.

The genetic background of varieties is also an important reason for the differences in citrus in low-temperature tolerance and the accumulation of off-flavor substances. Obenland (2013) analyzed the effect of variable temperature treatment (5 °C for 4 weeks and then 20 °C for one week) on the flavor quality of seven citrus varieties and found that deterioration in flavor was observed in four varieties (such as W. Murcott Afourer and Okitsu Wase), but not in the other two citrus varieties ('China S-9' and 'Gold Nugget'), which showed strong resistance and were not prone to developing off-flavors [3]. In this study, after being stored at 6–8 °C for 100 days and then transferred to room-temperature shelf life for one week (LTS_100d + RTS_7d), the ethanol content of 'Orah' mandarin was slightly above the threshold. However, after being stored at low temperature for 120 days and then transferred to room-temperature shelf life for one week (LTS_120d + RTS_7d), the ethanol content in 'Orah' mandarin increased sharply, far exceeding the threshold, and showed a strong off-flavor. Therefore, 100 days is the critical point of quality deterioration for 'Orah' mandarin when stored at a low temperature (6–8 °C). If the fruit is taken out of the warehouse and transferred to normal temperature shelves beyond this time limit, it will face the risk of irreversible flavor deterioration.

In addition to directly generating a large amount of ethanol and acetaldehyde, the metabolic products of fermentation metabolism can also be further derived as precursors into aldehydes, acids and ester compounds. The results from PCA analysis (Figure 5c) and clustering heatmap results (Figure 5e) jointly indicated that the LTS_120d + RTS_7d sample showed a similar profile of volatile compounds to that of LTS_180d and accelerated the accumulation of ethyl acetate and acetaldehyde in 'Orah' mandarin (Figure 5c). Furthermore, partial least squares discriminant analysis (PLS-DA) screened out ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, hexanoic acid and 1-pentanol as key DAMs, whose content significantly increased in the later stage of LTS (LTS_120d to LTS_180d) and during the LTS_120d + RTS_7d) (Figure 6e–g). These results indicated that treatment with LTS_120d + RTS_7d promoted the senescence process of 'Orah' mandarin. This result was consistent with the findings reported by Obenland et al., (2013) on W. Murcott Afourer [3] and He et al., (2014) on Newhall navel oranges [11]. This might be explained by the fact that, on the one hand, ethanol can react with endogenous organic acids in fruits through non-enzymatic reaction to form corresponding esters. On the other hand, the ethanol might be oxidized to acetaldehyde and further converted into acetic acid, which subsequently condenses with acyl-CoA (acyl-COA) to form the corresponding ester compounds by the catalysis from alcohol acyltransferase (AAT) [34,35]). The availability of ethanol as substrates is a limiting factor for the synthesis rate of esters [36]. In this study, the correlation heatmap and network diagram show that the content of ester substances was significantly positively correlated with the ethanol concentration (Figure 7). Similar results, that the content of ester substances was significantly positively correlated with the content of alcohols during post-harvest storage, were also observed in pear [37] and apple [36].

Esters with appropriate concentrations can significantly enhance the intensity and pleasantness of the fruit aroma [38–42]. Previous studies showed that the content of ethyl butyrate in kiwifruit is significantly positively correlated with the sensory attribute of fruit aroma, and a decrease in its content would directly lead to the loss of aroma [43].

Hexyl acetate and (E)-3-hexenyl acetate were significant contributors to the 'fruity' and 'pleasant' attributes in peach pulp. A decrease in their content directly lead to a notable weakening of these positive flavor attributes [44]. However, the concentration of ester compound did not show an infinitely linear increase relationship with its odor instead of a typical 'inverted U-shaped' dose–effect curve or concentration-dependent characteristic. At sub-threshold concentrations, esters mainly showed synergistic and additive effects which significantly enhanced floral and fruity aromas. Once the accumulation of esters exceeds the threshold value, this would result in a masking effect and even cause unpleasant flavors [45]. In this study, the explosive growth of ethanol in the 120d + 7d RTS pulp sample (4222.50 mg/L) drove the excessive synthesis of esters (Figures 5e and 6e–g), whose concentrations far exceeded the pleasure threshold, and instead, in synergy with the fermentation odor, produced unpleasant flavors such as a nail polish smell [45]. Similarly, studies have shown that storage at a temperature of 20 °C will cause abnormal accumulation of esters such as ethyl acetate, ethyl propionate, and 2-methylbutyrate in loose-skin citrus (mandarin), thereby resulting in unpleasant flavors [3]. Higher levels of ethyl acetate were detected in the 'Valencia' oranges that fell from the trees compared to the fruits remaining on the trees. Sensory evaluations indicated that the flavor of dropped fruit was often described as 'decayed' or with negative characteristics such as 'aftertaste bitterness'. The esters (especially ethyl acetate and propyl acetate) accumulated mainly as the products of anaerobic metabolism and were usually regarded as poor flavor indicators of fruit quality decline [46,47].

Taken together, the excessive accumulation of ethanol not only directly induced unpleasant flavors, but also served as a key precursor substance that drives the biosynthesis of short-chain fatty acids and ester-like odor derivatives. These effects of ethanol constituted the chemical basis for flavor deterioration during the shelf life. Based on the phased patterns of quality changes during low-temperature storage and on the shelf, precise intervention technical strategies should be formulated in stages. For example, in the middle and later stages of storage, the ethanol biosynthesis pathway should be inhibited by the likes of ethanol dehydrogenase (ADH) inhibitors or reducing anaerobic respiration of the fruit. During the stage connecting storage and sales, temperature fluctuations should be minimized as much as possible through cold chain or gradual rising of temperature.

5. Conclusions

RTS for 7 d after an LTS for 0 d, 20 d, 40 d, 60 d, 80 d, 100 d or 120 d exacerbated the decline of the pericarp chromatic a^* value, content of soluble solid, titratable acid, vitamin C, sucrose and malic acid in the pulp, as well as the accumulation of ethanol, methanol and acetaldehyde. Among the stages, the accumulation of ethanol in the sample from RTS for 7 d after an LTS for 120 d exceeded 2000 mg/L, resulting in a distinct unpleasant odor. In total, 43 volatile substances of seven categories including 1 alcohol, 1 aldehyde, 1 ester, 21 lipids/fatty acyls, 13 terpenoids, 4 organic acids, and 2 tetrahydropyrans were detected in the pulp. Continuous and steady decrease in the content of 1-octanol and terpenes (citronellol, (R)-(+)-α-terpineol, and β-linalool) within the first 60 days of LTS resulted in fast loss of aroma in the 'Orah' mandarin juice, while the substantial increase in ethanol, methanol and acetaldehyde (ethyl octoate, caproic acid) in the middle and later stages of LTS contributed to the prominent off-flavor. In conclusion, the LTS should not exceed 100 days due to the intensified accumulation of ethanol. The above results indicate that intervention techniques should be implemented in stages to inhibit the synthesis of ethanol in the middle of storage, and measures such as reducing temperature fluctuations should be used to block the rapid accumulation of ester compounds during the RTS. In the future, more in-depth research at the molecular regulatory level should be conducted to reveal the

molecular mechanism for the increase in off-flavors of 'Orah' mandarin and other kinds of citrus fruit during RTS after a LTS.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae12101202/s1, Table S1: Number of metabolites (from each class) that significantly correlated with physiological indicators; Figure S1: The appearance of 'Orah' mandarin during LTS and 7days of RTS; Figure S2: The weight loss of 'Orah' mandarin during LTS; Figure S3: The changes in content of sugars (sucrose and hexose) and organic acid (citric acid and malic acid) of 'Orah' mandarin and the effect of 7-days-room-temperature shelf on them. (**a**) sucrose & hexose; (**b**)citric acid & malic acid. Asterisks indicated significant difference between the LTS and the LTS+RTS at the same time point: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Figure S4: PLS-DA plot and model validation plot of pair-samples. (**a**) PLS-DA plot of LTS_60d vs LTS_0d; (**b**) model validation plot of LTS_60d vs LTS_0d; (**c**) PLS-DA plot of LTS_120d vs LTS_0d; (**d**) model validation plot of LTS_120d vs LTS_0d; (**e**) PLS-DA plot of LTS_120d+7d vs LTS_0d; (**f**) model validation plot of LTS_120d+7d vs LTS_0d; (**g**) PLS-DA plot of LTS_180d vs LTS_0d; (**h**) model validation plot of LTS_180d vs LTS_0d; (**i**) PLS-DA plot of LTS_120d vs LTS_60d; (**j**) model validation plot of LTS_120d vs LTS_60d; (**k**) PLS-DA plot of LTS_180d vs LTS_120d; (**l**) model validation plot of LTS_180d vs LTS_120d; (**m**) PLS-DA plot of LTS_120d+7d vs LTS_120d; (**n**) model validation plot of LTS_120d+7d vs LTS_120d; Figure S5. Venn analysis of differential accumulated metabolites. (**a**) Venn analysis of differential accumulated metabolites from LTS_60d vs LTS_0d, LTS_120d vs LTS_0d, and LTS_180d vs LTS_0d. (**b**) Venn analysis of differential accumulated metabolites from LTS_60d vs LTS_0d, LTS_120d vs LTS_60d, LTS_180d vs LTS_120d and LTS_120d+RTS_7d vs LTS_120d. Note: LTS_0d represent as 0DAH; LTS_60d represent as 60DAH; LTS_120d represent as 120DAH; LTS_180d represent as 180DAH; LTS_120d+RTS_7d represent as 120DAH_7D.

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