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Postharvest exogenous melatonin treatment of strawberry reduces postharvest spoilage but affects components of the aroma profile

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ABSTRACT

Background: Strawberries are perishable fruits that decay quickly after harvest, but are valued for their distinctive taste and aroma. Melatonin is involved in plant resistance against stress, plant senescence and fruit ripening, and was shown to delay post-harvest spoilage of strawberries.

Objective: The effects of melatonin postharvest treatment on shelf-life and volatile organic compound profile were assessed in strawberry fruits cv "Luca".

Methods: Strawberry fruit were treated with 100 µM melatonin and stored at 4 °C for 12 days to assess whether melatonin treatment could delay spoilage without adversely affecting aroma.

Results: Melatonin treatment delayed fruit deterioration by reducing weight loss and incidence of decay as well as maintaining total soluble solids, titratable acidity, anthocyanin, and taste. Melatonin treatment also significantly reduced CO_2 production compared to control fruits. The relative abundance of the majority of volatile organic compounds (VOCs) was not affected, however abundance of two VOCs that are important components of strawberry aroma were affected by melatonin treatment.

Conclusions: Post-harvest treatment of strawberries with 100 μM melatonin improved strawberry quality and conserved bioactive compounds after 12 d of storage. However, components of the aroma profile were altered in a way which may affect consumer perception of quality.

Key words: ethyl hexanoate, *Fragaria×ananassa*, melatonin, postharvest quality, volatile organic compounds.

1. Introduction

North and West African countries are considered as the main suppliers of fresh fruits and vegetables to northern Europe and most EU countries [1]. This supply chain requires long periods of transport and storage under controlled refrigerated conditions, which can result in the proliferation of postharvest spoilage microbiota [2], as well as fruit deterioration through bruising. Two major problems are linked with the globalised supply chain for fruit and vegetables: postharvest loss of produce and the dispersal of human pathogens with produce.

Strawberries are a high value crop, rich in bioactive compounds of known health benefit. These include vitamins, especially vitamin C and E, and other bioactive compounds such as β-carotene and phenolic compounds [3]. However, as is the case with all soft fruit, strawberry shelf life is very limited, leading to substantial waste in the supply chain [4]. Strawberries are considered as one of the most non-climacteric perishable fruits, with a very limited shelf life [5]. There is therefore a need for a treatment which stabilises strawberry quality after harvest and prolongs shelf life.

Melatonin is produced by all eukaryotes including strawberry fruit [6] and is a potent antioxidant. Moreover, exogenous treatment of plants with melatonin can mitigate effects of abiotic stress by reducing reactive oxygen species and interacting with hormone signalling [7]. Melatonin can also be applied to fruit post-harvest. Exogenous postharvest melatonin application at 0.1 or 1.0 mmol L^{-1} to strawberry cv. Hongyan fruit delayed fruit senescence, reduced decay and weight loss, while total phenolics increased, resulting in higher antioxidant content [8]. Similarly, exogenous 100 μmol L^{-1} melatonin treatment resulted in a reduction in decay and increased total phenolic compounds and anthocyanins in strawberry cv. Selva fruits [9]. However, little is known about the effects of melatonin on VOCs, key components of the aroma. An alteration in flavour-related VOCs would have implications for the use of melatonin as a treatment to improve quality and postharvest shelf-life of the fruit. We hypothesised that the effects of melatonin treatment might affect the profile of volatile compounds.

2. Materials and methods

2.1. Plant material, melatonin treatments and storage conditions

Strawberry fruit (*Fragaria*×*ananassas* Duch., cv. Luca) were collected at the commercial maturity stage (98 % red colour, assessed visually, and 4.5 week after flowering) from a local farm (Haygrove, Cardiff). The fruits were immediately transported to Cardiff University, School of Biosciences, Wales, UK. Fruit were then carefully sorted to exclude misshapen, overripe, underripe or damaged fruit. The experiment began on the same day. The sorted fruits were randomly divided into three homogenous groups of 300g, representing the number of treatments. Strawberries were immersed in three different solutions for 5 min: control (distilled water), 50 μM or 100 μM melatonin in distilled water. Following immersion, the fruits were dried for 2 hours at room temperature (RT) in a sterile flow hood. The fruits were placed in polyethylene trays (clamshells), then stored for 12 days at 4 °C and 90 % relative humidity. The experiment was repeated twice, with three replicates for each treatment in each of the two experiments. Since no significant differences between 0 and 50 μM concentrations of melatonin were found in any of the parameters tested, results are only presented for the control and 100 μM melatonin treatments.

2.2. Weight loss and decay assessments

To determine weight loss, strawberry fruits were weighed immediately after air-drying and at every sampling time. The results are shown as the percentage weight loss compared to the initial fresh weight.

For evaluation of decay, decay percentage (%) was calculated by weighing all decayed fruits (affected by visible signs of rot or fungal growth) relative to the weight of total fruits. Fruits free from any decay were used for further analyses. The fruits were sliced in small pieces and immersed immediately in liquid nitrogen and stored at −80 °C until used to measure titratable acidity (TA), anthocyanin and phenolic content.

2.3. Analysis of colour, total soluble solids (TSS), pH, and titratable acidity (TA)

The colour of the strawberry surface was measured with a digital camera (Cannon, Japan). Six fruits per replicate were used for the colour test. Images were calibrated using Image J software.

TSS in strawberry juice was measured using a refractometer (RHB-18ATC). The TSS reading (in degree Brix, Bx) was expressed as the % of TSS in the fruit.

To measure TA, three fruits form every replicate were homogenized with a tissue homogenizer for 5 min then 5 g of strawberry fruit juice was diluted to 50 mL with distilled water and titrated to pH 8.1 using 0.1 M NaOH. TA was calculated according to AOAC [10] and expressed as percentage of citric acid equivalent, since citric acid is the principal acid in strawberries [11]. The pH of the juiced strawberry fruit was measured with a calibrated pH meter (EuTech, Instruments, pH 510, Singapore).

2.4. Taste index

Taste index (TI) was calculated with the equation below [12] using the TSS and TA values:

TI = (TSS value/20×TA value) + TA value

2.5. CO_2/O_2 emission rate and ethylene production rates

To measure the CO_2/O_2 emission rate, individual strawberry fruits were placed in 250 mL sealed glass containers and after 1 h of enclosure at room temperature (20 °C), 1mL of air sample was extracted from the headspace and analyzed using a gas analyser (Model ML206, AD-Instruments, Australia) for CO_2/O_2 ratio.

To measure ethylene production, strawberry fruits were incubated in a 250 mL super sealed glass jar for 60 min at room temperature (25 °C). Each strawberry fruit sample was weighed using an analytical balance to record its initial weight. The caps of the containers were drilled (1 mm in diameter), to enable head space gas sampling. A rubber seal was inserted to cover the hole to prevent the gas inside the container leaking. One mL of the headspace was sampled from the glass jar via an air tight syringe (SGE, Analytical Science, PA, USA), and injected immediately into a gas chromatograph (Agilent Technology, 6890N GC system, USA) fitted with an Alumina Sulfate Plot column (30 m x 0.32 mm, Supelco) column and a FID detector. Column temperature was 70 °C and injection temperature was 120 °C. Helium was used as a carrier gas with a flow rate of 1 mL min^{-1}. The rate of ethylene emission was expressed as μl C_2H_4 kg^{-1} FW h^{-1} using ChemStation software (Rev.A.09.01, Agilent technologies, USA).

2.6. Collection and analysis of Volatile organic compounds (VOCs)

At day 0 and 4 of cold storage at 4 °C, the lids of the containers were removed, the samples were sealed into a 25 cm x 38 cm nalophene plastic bag (TJM Ltd), and they were equilibrated for 2 hours at room temperature (20 °C). Headspace gas (400 mL) was collected using a hand pump (Easy VOC pump, Markes International Ltd.) onto thermal desorption tubes packed with Tenax TA and SulfiCarb sorbents (Markes International Ltd.).

The VOCs collected for both experiments were then analysed after thermal desorption by gas chromatography and time of flight mass spectrometry (TD-GC-TOF-MS) essentially as described by Spadafora et al. [13]. A TD100 (Markes International Ltd.) was used to desorb tubes and inject samples into the GC. Samples were desorbed onto the trap (at 25 °C) first for 5 min at 120 °C and then for 5 min at 260 °C with 40 mL min^{-1} nitrogen. The trap was desorbed at 300 °C for 3 min with 40 mL min^{-1} helium resulting in a split ratio of 20:1 into the GC (7890A, Agilent Technologies, Inc.). VOCs were separated on a 60 m, 0.32 mm I.D. and 0.5 μm film thickness Rxi-5ms capillary column (Restek). The temperature program was: 40 °C for 5 min, 10 °C min^{-1} ramp to 300 °C, final hold 5 min (total run time 41 mins). A BenchTOF-dx MS (Almsco International) was used to detect VOCs. It was operated at a source temperature of 275 °C and filament voltage of 1.6 V. Ions were collected in the mass range 35-500 m/z. A retention time standard (C8-C20, Sigma Aldrich) was run with each set of samples and prepared by injecting 1μl of the standard mixture directly onto a TD tube (Tenax TA).

Data was processed and analysed using AMDIS (NIST 2014) and MSD ChemStation software (E.02.01.1177, Agilent Technologies, Inc.). A custom MS library was produced using retention indices (MS spectra were searched against the NIST library with over 80 % identification in forward and backward fit). Putative identification was made on the basis of >80 % match of mass spectra and +/-15 in retention index to the custom library. These were then processed to remove contaminants, defined as compounds not present in at least two replicates, and compounds present at similar levels in control samples.

2.7. Total anthocyanin content

Total anthocyanin was measured according to previously published methodology [14]. Fruit pulp (2 g) was blended with extraction solvent (20 mL of ethanol, 1.5 N HCl, 85: 15) and kept overnight at 4 °C. The samples were then filtered into a volumetric flask and covered with aluminium foil. The remaining residue was washed with extraction solvent until the pigments were removed. Filtrates were pooled and made up to 100 mL with extraction solvent. Absorbance was recorded at 535 nm to determine the anthocyanin content using the following formula: Absorbance at 535 nm × volume of extraction solution × 100/ weight of sample × 98.2. The results are expressed as mg/100 g fresh weight.

2.8. Total phenolic content

Strawberry fruits from each treatment were homogenized using a laboratory blender. The mixture was centrifuged at 5000 g for 20 min at room temperature. The supernatant was filtered through a paper filter (Whatman N. 1) to yield a clear juice. Total soluble phenolics were measured using Folin–Ciocalteu reagent according to published methodology [15]. Briefly, 0.5 mL juice aliquots were diluted in 9.5 mL distilled water. Then, to 1 mL of the resulting solution, 5 mL of a diluted (1 + 9 distilled water) Folin–Ciocalteu reagent (Fisher Scientific International, Leicestershire, UK) were added. Then 4 mL of sodium carbonate solution 7.5 % (BDH Limited, Poole, England) were added and after 1 h at 30° C and 1 h at 0° C, the absorbance of the solution was measured at 760 nm with a model SP8-400 UV/VIS Spectrometer (Pye, Unicam Ltd, Cambridge, England). Using gallic acid (97-5.102.5 % (titration) Sigma Aldrich, China) as the standard. Results are expressed as mg of gallic acid equivalents (GAE) per L.

2.9. Statistical analysis

All physiological and biochemical data were analysed statistically using a one-way ANOVA test, where *P*-values < 0.05 were considered significant, using SPSS 19 statistical software. The mean values ± SE were compared using a Tukey test. Changes in individual VOC abundance at each time point were analysed using a Student’s t-test. Analysis of total VOC profiles was performed essentially as described in Spadafora et al. [13]. Peak areas were normalised to the total area of the chromatogram for each sample, and the square root of the area was used for further analysis to reduce the contribution of larger component areas. Using the R platform (version 3.1.3; R core development team 2015) data were analysed using PerMANOVA (Permutational Multivariate Analysis of Variance) and CAP analysis (Canonical Analysis of Principal coordinates) tests [16]. These were carried out using the 'vegan' and BiodiversityR' packages within R. This analysis treats the whole profile ans a single independent variable. An ordination plot was generated by the software and a 95% confidence interval was fitted to the data.

3. Results and Discussion

3.1 Weight loss and decay are significantly affected by treatment with melatonin

Melatonin treated fruits lost significantly ($P < 0.05$) less weight than the controls starting from 2 days of storage, with the difference increasing until the end of the storage period (Figure 1A). Moreover, overall weight loss decreased significantly by 59.76 % in melatonin treated compared with control fruits. There was no decay observed until day 4 of storage (Figure 1.B; Supplementary Table 1). After this, decay was reduced in the melatonin treated fruits both at days 8 and 12 of storage, and by 50.01% compared to the controls at 12 days of storage. Similar effects of melatonin were shown previously on decay in strawberry[11] and on both decay and weight loss in peach [17]. The reduced decay of the strawberry fruits elicited by the 100 μM melatonin treatment could be due to its effects on reactive oxygen species resulting in increased cell wall rigidity. Higher superoxide dismutase enzyme activity in parallel with lower ascorbate peroxidase and catalase enzyme activity in fruits was shown to result in H_2O_2 accumulation as a result of melatonin treatment of strawberry fruit [9]. This in turn activated enzymes in the phenylpropanoid pathway, which enhance cell wall rigidity and the nutritional quality of the fruit. Calcium also plays an important role in cell wall structure and tissue firmness. Melatonin application led to a reduction in water loss and an increase in calcium content in maize seedlings under cold stress [18]. The weight loss and reduced decay elicited by melatonin treatment in chilled strawberry fruit shown here could therefore possibly be due to redistribution of the calcium in strawberry fruit tissue leading to less moisture loss. Post-harvest dipping in calcium solutions and thus increasing calcium content is known to decrease the decay of strawberry fruits [19].

3.2 Taste index was improved by melatonin treatment: while total soluble sugars increased, titratable acidity was unaffected

Total soluble solids content (TSS) increased slightly but significantly ($P < 0.05$) from 0 to 2-4 days of storage and decreased thereafter in both treatments (Supplementary Table 1). Fruits treated with 100 μM melatonin had a significantly higher TSS than the control after 4 and 8 days of storage, whereas at the last storage time point, there was no significant difference between treatments. Our results are in accordance with Gao et al. [17] who reported that melatonin treatment significantly increased retention of TSS content during cold storage of peach fruits. In addition, tomato plants supplemented with melatonin showed significant increases in their contents of TSS in fruits [20].

There was no significant difference in TA between 100 μM melatonin treated and control strawberry fruits until 4 days of storage (Figure 2A). However, after 8 and 12 days of storage, TA was significantly higher in the melatonin treated fruits than in the controls. Previous work [20] reported that tomato plants irrigated continuously with melatonin had significantly higher content of citric acid in fruits. They suggested that organic acids were enhanced by melatonin treatment. The pH of strawberry fruits was significantly higher in the control than in the treated fruits at the end of the storage period (Figure 2.B), however, there was no significant difference between the two treatments at other time points.

The taste index (%) of melatonin treated fruits was significantly higher ($P < 0.05$) than control fruits at 8 and 12 days of storage (Figure 2C and Supplementary Table 1). The higher taste development induced by melatonin treatment might be due to a general increase in total sugars and organic acids [21].

3.3 Treatment with melatonin affected anthocyanin and phenolic content at selected storage time points

Anthocyanin increased significantly ($P < 0.05$) with storage time (Supplementary Table 1). The treatment with melatonin increased anthocyanin content in the fruits compared to the control from 4 days until 8 days of storage, while there was no significant difference at the end of the storage period. Previously, Aghdam and Fard [9] found higher anthocyanin content in strawberry fruits treated with melatonin compared to the control. They proposed that this may be due to the higher phenylalanine ammonia lyase (PAL) enzyme activity which leads to accumulation of anthocyanins.

Total Phenols decreased up to 4 days of cold storage then slightly increased ($P < 0.05$) Figure 3B and Supplementary Table 1). Total phenols were significantly higher ($P < 0.05$) in fruits treated with melatonin than in control fruits at 2 and 4 days of storage, however there were no differences at 8 and 12 days between treatments. Similar results were previously reported in strawberry [8] indicating that total phenol content is significantly increased by melatonin treatment in strawberry fruit. The increase in total phenol contents in strawberry fruit might be explained by a higher phenylalanine ammonia lyase (PAL) enzyme activity induced by the melatonin treatment leading to an accumulation of phenols [9].

3.4 CO_2 production increased with melatonin treatment while ethylene production and the overall profile of volatile organic compounds was unaffected

CO_2 production rate, which is considered here as a proxy for the respiration rate, was significantly higher in control fruit than melatonin treated fruits throughout storage until the last time point (Figure

3C). However, there were no statistically significant differences observed in ethylene production (Supplementary Table 2). Strawberry fruit is considered non-climacteric and produces a low level of ethylene [22] which is consistent with the results seen here. However, the relationship between respiration rate and melatonin treatment is unclear: the increase in respiration seen in the melatonin treated fruit could be due to the role of melatonin in plant metabolism and an alteration to the balance of plant growth regulators [23] however further studies are needed.

Fifty volatile organic compounds (VOCs) were identified from all the samples including esters (total 33 with eight acetate and 25 non-acetate), aromatic compounds (5), alkanes (3), furans (3), ketones (2), terpenes (2) and nitriles (1) (Supplementary Table 3). Analysis using PerMANOVA indicated that the overall VOC profile changed across time $P < 0.001$, $R^2 = 0.470$). Linear discrimination plots based on CAP analysis separated the two storage time points with 100% correct classification (Figure 4). However, there was no discrimination between melatonin treated and non-treated fruits (Supplementary Table 4). A closer examination of changes in individual VOCs in treated vs. control fruit at the two time points reveals that indeed the majority of VOCs did not change in abundance significantly. However ethyl 2-methylbutanoate was absent from fruit treated with melatonin at day 0 while it was present in the control fruit, and ethyl hexanoate was 2-fold more abundant in the headspace of control fruit after 4 days of storage compared to fruit treated with melatonin ($P < 0.05$; Table 1; Supplementary Table 4). Both VOCs have been reported as important components of strawberry bouquets previously [24], however their abundance varied across different cultivars and abundance of ethyl hexanoate also increased with storage. Further sensorial analysis would be required to assess whether the changes noted here in VOC profile following melatonin treatment significantly change the perceived aroma of the fruit.

4. Conclusion

We confirmed previous work showing that exogenous treatment of strawberry fruit with melatonin at 100 µM would delay fruit deterioration. In fact, it decreased weight loss, decay and respiration rate during cold storage at 4° C. In addition, the treatment increased TSS, anthocyanin, and total phenols compared to the control. However, melatonin treatment did not affect ethylene production or colour intensity. Although overall volatile organic compound profile was not affected by the melatonin treatment, the abundance two important aroma related VOCs was affected. The quality of strawberry could therefore be enhanced by treatment of melatonin however the changes to the aroma may affect consumer quality assessments of the fruit. Further studies are needed to fully understand the mechanism of melatonin action on non-climacteric fruits, and the organoleptic effects on fruit aroma.

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Compliance with ethical standards

Conflict of interest statement

The authors declare no conflict of interest.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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TABLES

Table 1 Relative abundance of two VOCs that were significantly affected by the melatonin treatment

VOC	Ratio of relative abundance between mealotinin treated and untreated fruit (± SD)*

	Day 0		Day 4	
	control	+MT	control	+MT
Butanoic acid, 2-methyl-, ethyl ester	0.362± 0.05 ^{a}	0 ^{b}	0.03± 0.06 ^{a}	0.02± 0.04 ^{a}
Hexanoic acid, ethyl ester	17.3± 2.38 ^{a}	21.9± 4.99 ^{a}	5.94± 1.38 ^{a}	2.80 ± 0.74 ^{b}

*different letters indicate significant differences (*P* < 0.05) based on a student t-test between treated and untreated samples

FIGURE LEGENDS

Fig 1: (A) Weight loss, (B) decay, and (C) TSS of strawberry fruits treated postharvest with 0 and 100 µM melatonin and stored at 4 ± 0.5 °C for up to 12 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Different letters indicate significant differences between melatonin treated and control fruit at each time point (Tukey test at *P* < 0.05).

Fig 2: (A) Titratable acidity, (B) pH, and (C) Anthocyanin of strawberry fruits treated postharvest with 0 and 100 µM melatonin and stored at 4 ± 0.5 ℃ for up to 12 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Different letters indicate significant differences between melatonin treated and control fruit at each time point (Tukey test at *P* < 0.05).

Fig 3: (A) CO_2, (B) total phenol, and (C) taste index of strawberry fruits treated postharvest with 0 and 100 µM melatonin and stored at 4 ± 0.5 °C for up to 12 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Different letters indicate significant differences between melatonin treated and control fruit at each time point (Tukey test at *P* < 0.05).

Fig. 4. Canonical Analysis of Principal ordinates related to time of storage based on all 50 strawberry VOCs using TD-GC-TOF-MS**:** Each ellipse represents the 95% confidence interval. The plots use

linear discriminants LD1 and LD2 with a percentage of correct classification of 100% and $P < 0.001$ (n=6).

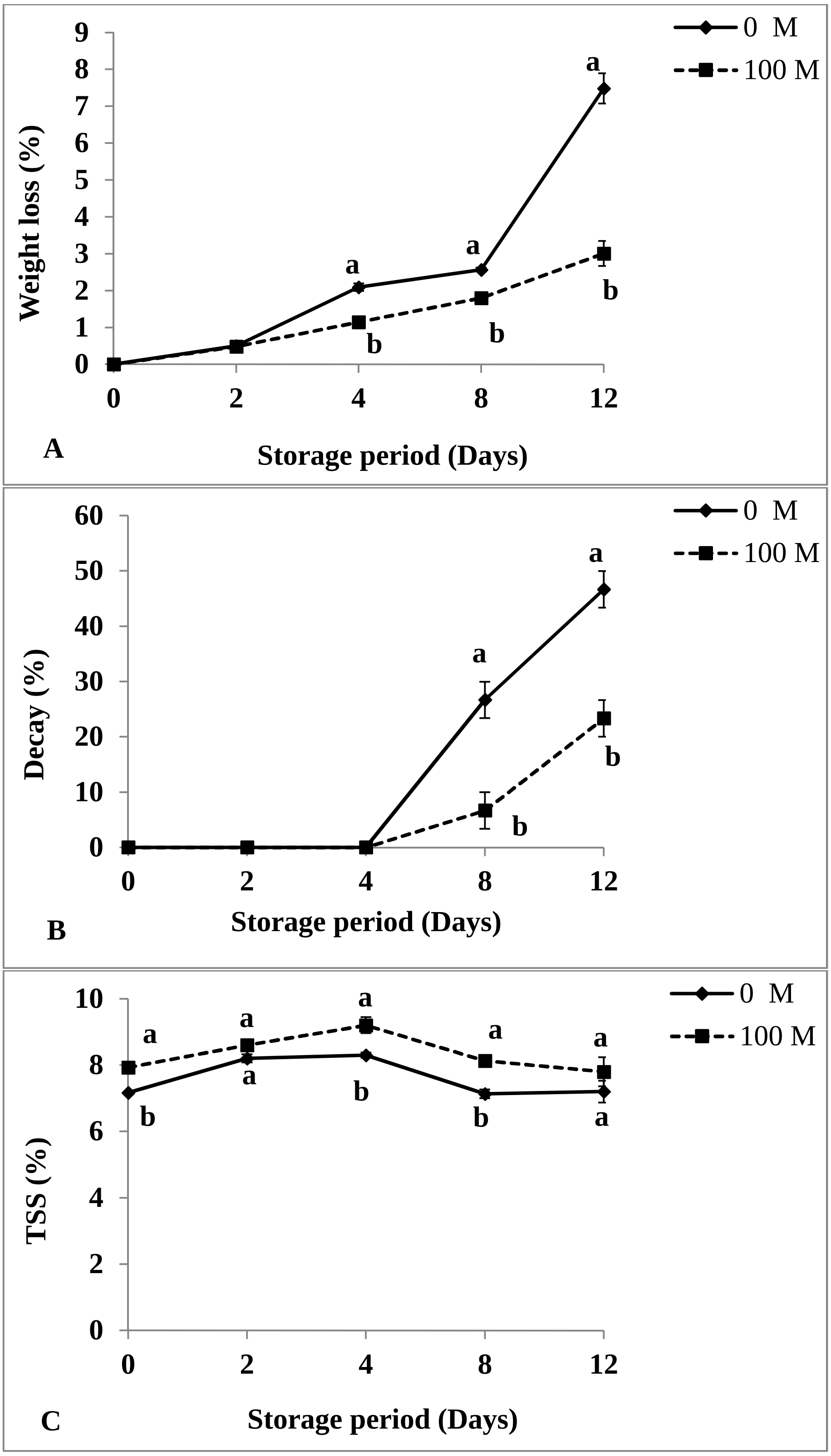
SUPPLEMENTARY TABLES

Supplementary Table 1: Statistical analysis of effect of storage duration on physical and chemical parameters.

Supplementary Table 2 - Emission of ethylene (µl kg-1 h-1) from strawberry fruit treated with melatonin and controls.

Supplementary Table 3 - Volatile organic compounds detected in all strawberry fruit samples

Supplementary Table 4 - % abundance of each VOC across 3 replicates of each treatment at each time point including fold change and significance of change.



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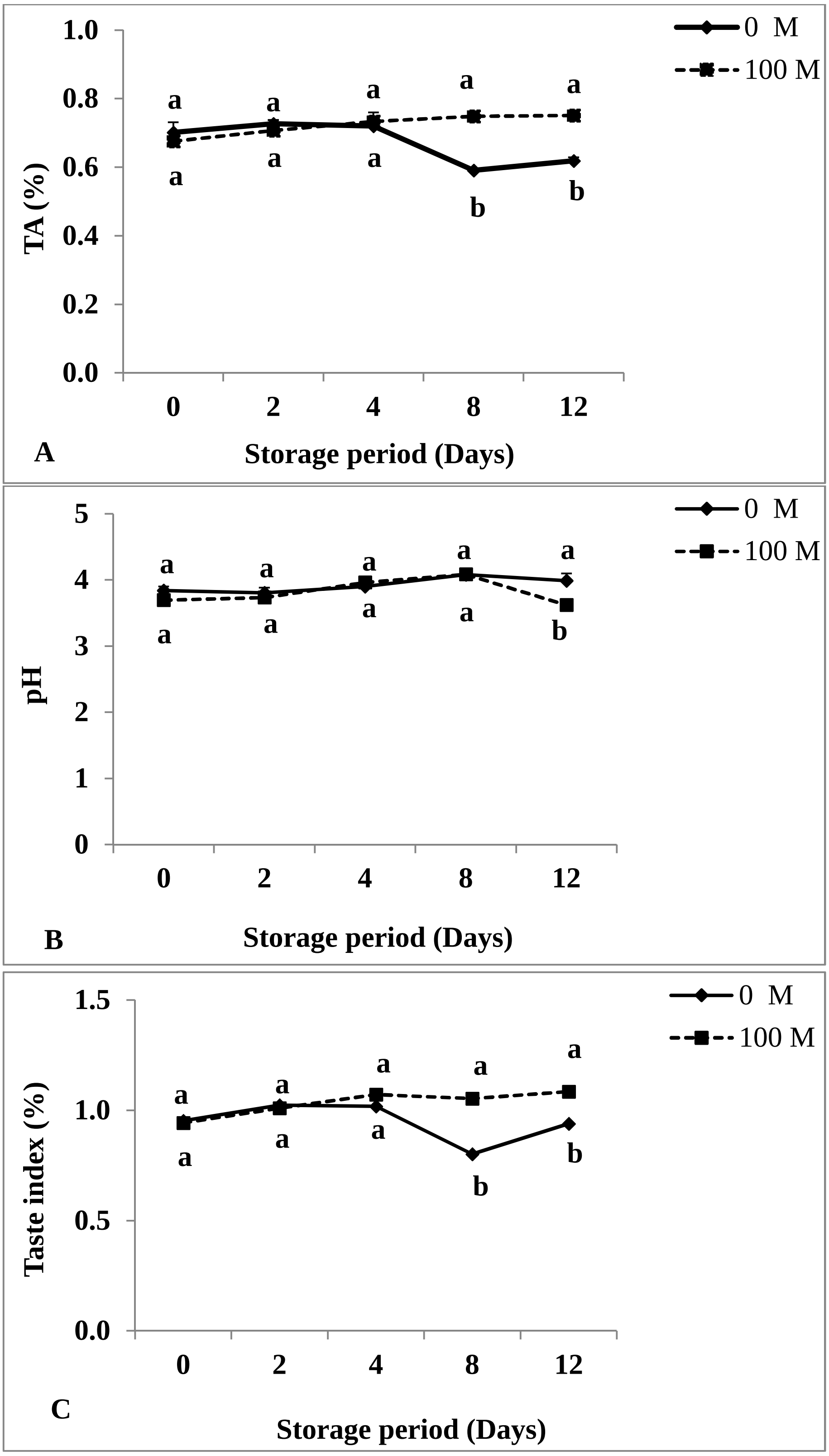

Fig 2: (A) Titrable acidity, (B) pH, and (C) taste index of strawberry fruits treated with postharvest 0 and 100 μM melatonin and stored at 4 ± 0.5 °C for up to 12 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Different letters indicate significant differences between melatonin treated and control fruit at each time point (Tukey test at $P < 0.05$).

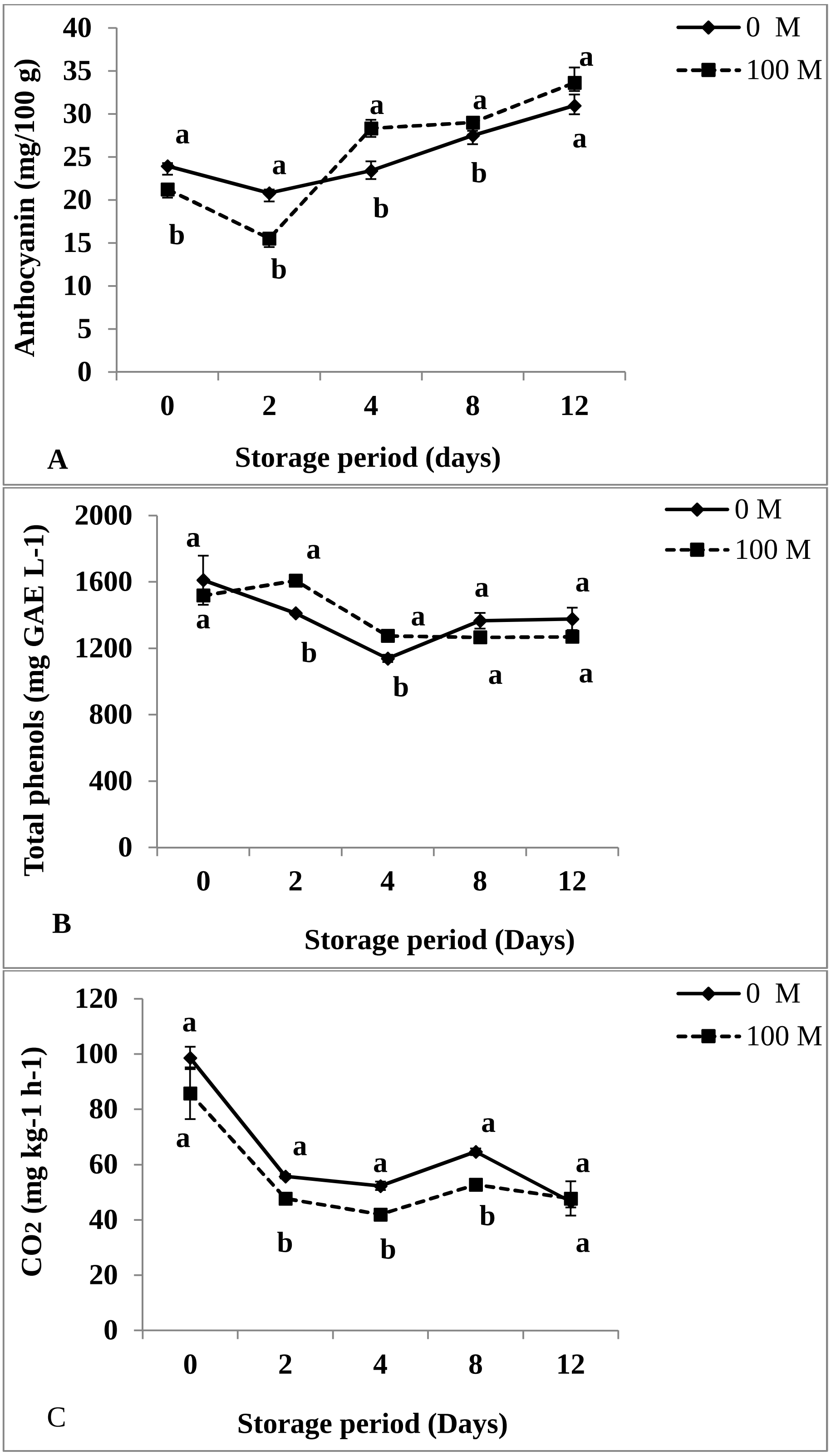

Fig 3: (A) Anthocyanin, (B) total phenols, and (C) CO_2 of strawberry fruits treated with postharvest 0 and 100 µM melatonin and stored at 4 ± 0.5 °C for up to 12 days. Data shown are mean values of n = 3 and the error bars represent standard errors of the means. Different letters indicate significant differences between melatonin treated and control fruit at each time point (Tukey test at $P < 0.05$).

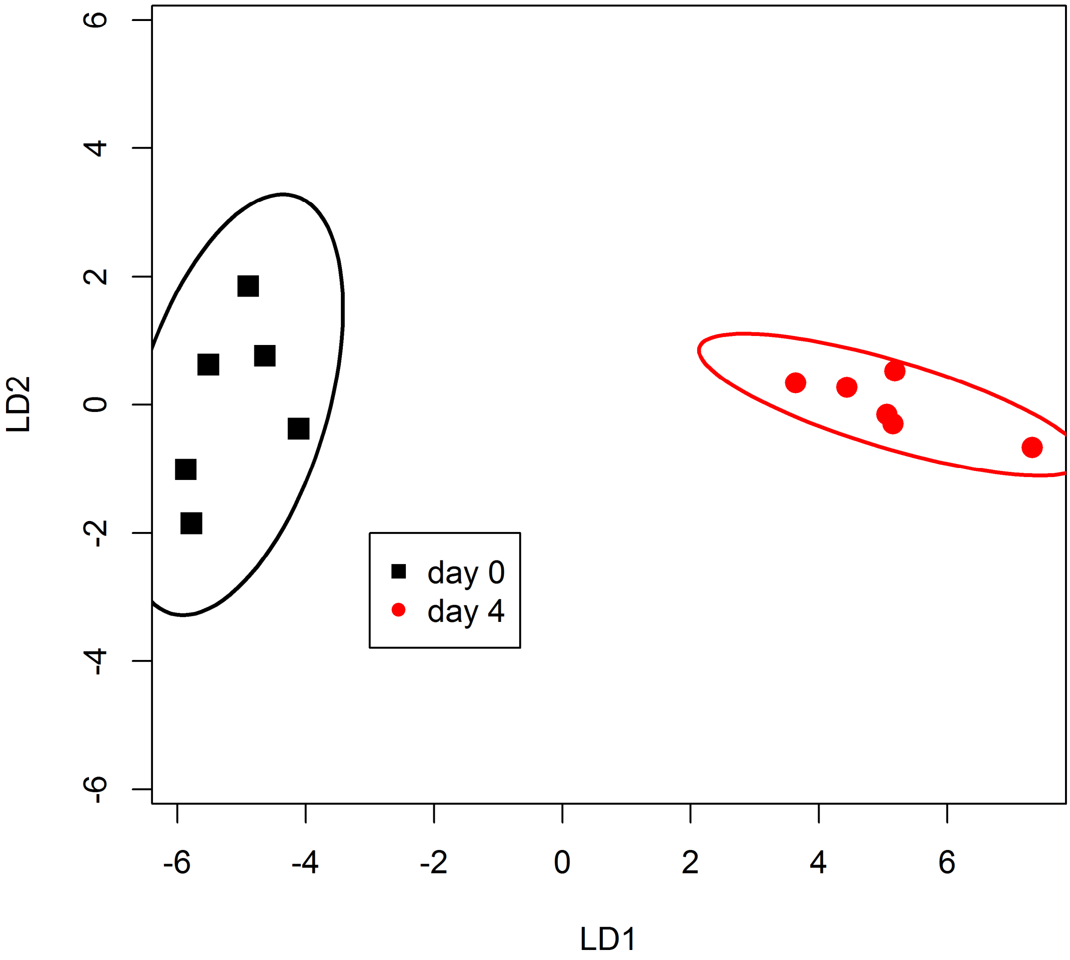

Fig. 4. Canonical Analysis of Principal coordinates related to time of storage based on all strawberry VOCs using TD-GC-TOF-MS**:** Each ellipse represents the 95% confidence interval. The plots use LD1 and LD2 with a percentage of correct classification of 100% and $P < 0.001$ (n=6).