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Modified Atmospheric CO₂ Levels for Maintenance of Fruit Weight and Nutritional Quality upon Long-Term Storage in Blueberry (*Vaccinium corymbosum* L.) ‘Liberty’

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Abstract: Blueberry fruits have gained consumer attention in recent years due to their good taste and high nutritional value. However, the short shelf-life of the fruit is one of the main downsides in intensive blueberry production. Therefore, optimized storage technology with a modified atmosphere is necessary to prolong blueberry fruit quality on the market. The aim of this study was to investigate long-term storage of fruit of the highbush blueberry (*Vaccinium corymbosum* L.) ‘Liberty’ under the air control (0.5% CO₂, 19.5% O₂, 80% N₂) and controlled atmosphere conditions of: 5% CO₂, 5% O₂, 90% N₂; 15% CO₂, 5% O₂, 80% N₂; and 25% CO₂, 5% O₂, 70% N₂. Fruit sampling was performed four times during storage (17, 30, 44, 62 days). Evaluation was carried out for fruit weight, total and individual sugar and organic acid contents, sugar-to-organic acid ratio, and individual phenolics contents. After 44 days of storage, weight loss was highest with 15% CO₂ and lowest with 5% CO₂, with minor variations. The greatest breakdown of total sugars was seen for the air control, and the least for 25% CO₂. Organic acids were significantly reduced under all of these storage conditions. Consequently, a high sugar-to-organic acid ratio was maintained in fruit stored with 25% CO₂. The contents of all of the identified phenolics significantly decreased with 15% and 25% CO₂. After 62 days of storage with 5% CO₂, there were small decreases in flavan-3-ols and hydroxycinnamic acids, while flavonoid and anthocyanin contents were unchanged, or for some individual phenolics, content increased. These data show that 15% CO₂ or higher accelerates degradation of the phenolics. We can conclude that for maintenance of weight and nutritional quality of the blueberry fruit ‘Liberty’, the optimal controlled atmosphere under long-term storage is 5% CO₂, 5% O₂, and 90% N₂.

Keywords: highbush blueberry; controlled atmosphere; weight loss; sugar-to-organic acid ratio; phenolics

1. Introduction

Consumer demand for blueberry (*Vaccinium corymbosum* L.) fruit has risen considerably in recent years due to their recognizable taste, good nutritional value, and potentially beneficial effects on human health [1–3]. Blueberry fruit are rich in phenolics, vitamins (i.e., A, B1, B2, C), and carotenoids. Anthocyanins are the most abundant group of phenolics in ripe blueberry fruit, followed by flavonols, phenolic acids, and flavan-3-ols. Altogether, these phenolics provide blueberry fruit with high antioxidant potential [1,4,5]. Data published to date shows that long-term ingestion of fruit rich in phenolics, such as blueberries, can protect against cardiovascular diseases, stroke, high blood pressure, and osteoporosis [6,7].

Blueberry fruit are seasonal, and due to their high popularity, most countries must import them, and so their arrival on the market can take from several days to several

weeks [8]. At the same time, once on the market, blueberry fruit last for a relatively short period of time, due to rapid quality deterioration under the presently used storage conditions. During storage, blueberry fruit can be subject to postharvest decay, water loss, physiological breakdown, decline in sensory quality, and fungal contamination [9,10]. These effects depend on the storage conditions (e.g., temperature, relative humidity, gas atmosphere), the stage of ripeness at harvest, the methods used for harvest and fruit transport, and the presence of pathogens [11]. At the same time, bioactive compounds can decompose rapidly due to oxidation, which is based on oxygen exposure [12].

In addition to cultivar selection, a storage method that prolongs the shelf-life of these fruit would be desirable. To extend the shelf-life to 18 days, blueberry fruit should be stored at 0 °C and a relative humidity from 90% to 95% [13]. This also helps to maintain the high phenolics content, although this is a relatively short period of time. A controlled atmosphere under modified O₂ and CO₂ conditions (e.g., elevated CO₂ and/or reduced O₂) is commonly used for fresh fruit transportation, and to a lesser extent for storage, to maintain fruit quality and avoid spoilage [14,15]. An appropriate O₂ level is necessary to avoid oxidation, or if this is below the tolerance threshold of the fruit, fermentation [8].

Duarte et al. [16] and Catuneanu et al. [17] reported differences in blueberry cultivar responses to the same storage conditions, based on the individual sugars and organic acids (i.e., primary metabolites) and phenolics (i.e., secondary metabolites) in the fruit during storage. CO₂ at 5% provided the greatest benefits for fruit quality of the 'Brigitta' [16], and 5% or 10% CO₂ for the 'Coville', 'Blueray', and 'Chandler', depending on the compounds of interest [17].

Blueberry storage technology with a non-modified atmosphere prolongs the shelf-life of blueberry fruit for less time than desired. To maintain the nutritional quality of the fruit for several weeks instead of days, the storage technology needs to be optimized. Therefore, the purpose of the present study was to determine for the blueberry fruit 'Liberty': (1) how much the fruit quality changes during storage; (2) which CO₂ concentration for storage has the least negative effects on the fruit from the point of view of the primary and secondary metabolites; (3) which storage technology preserves the fruit weight and chemical composition to the greatest extent; and (4) for how long can the fruit be stored under individual gaseous conditions to maintain high sugar-to-organic acid ratio and phenolics content.

2. Materials and Methods

2.1. Plant Material and Harvest

The blueberry (*Vaccinium corymbosum* L.) bushes of 'Liberty' were planted in 2013 in a test field at the Agricultural Institute of Slovenia in Brdo pri Lukovici (latitude, 46°10' N; longitude, 14°41' E; altitude, 380 m a.s.l.), with separation of 1.3 m × 3.0 m. These were grown according to the integrated production guidelines, on a silty loam soil with high potassium and nitrogen and low phosphorus levels. 'Liberty' was chosen for its aromatic taste and due to its increase in planting area. The orchard was covered with a hail net from the end of flowering until the end of ripening, and was equipped with a drip irrigation system. For the experiment, 15 uniform plants of this blueberry fruit 'Liberty' were selected. The fruit harvest was conducted by hand at commercial maturity, full size, and when the fruit were fully blue at least 5 days before harvesting. The blueberry fruit were harvested on the morning of 1 August 2018, using transparent polystyrene baskets with a 1 kg capacity, and then immediately transported to cold storage conditions.

2.2. Storage Conditions

After the harvest, all of the fruit were immediately cooled down to 1 °C (±0.5 °C) over 3 h, to prevent condensation. After cooling, 100 g of the fruit was weighed and put into smaller perforated plastic baskets (i.e., as also used when sold). All of the samples were packed and heat-sealed in polyethylene bags (non-permeable polyamide/polyethylene plastic bags, PA/PE/PE-105 µm), to prevent the controlled atmosphere from mixing with the outside atmosphere.

For the fruit storage, 48 bags were prepared (i.e., 12 for each condition), and all were stored at 1 °C (± 0.5 °C) and 90% to 95% relative humidity (based on previous studies; ¹³). These samples were sealed and stored as the air control (0.5% CO₂, 19.5% O₂) and under the following three controlled atmosphere conditions: 5% CO₂, 5% O₂, 90% N₂; 15% CO₂, 5% O₂, 80% N₂; and 25% CO₂, 5% O₂, 70% N₂. These CO₂ concentrations were chosen based on previous studies [16] and because 10% CO₂ has been used recently in storage with a modified atmosphere. These gas concentrations were achieved by ventilating the bags with the appropriate (already prepared) gas mixture, as defined above. The individual atmospheres were monitored using a CO₂ sensor (Geosensor-G100; Geotechnical Instruments Ltd., Coventry, UK) and the bags were sealed when the desired CO₂ concentration was reached.

The first sampling date was 18 August 2018 (after 17 days storage), the second was on 31 August 2018 (30 days storage), the third was on 14 September 2018 (44 days storage), and the fourth (and final) was on 2 October 2018 (62 days storage). At each sampling date, three baskets of fruit in the bags under the individual atmosphere conditions were removed, and the fruit weights were recorded for each. The fruit were then frozen in liquid nitrogen and stored at -80 °C until extraction of the individual sugars and organic acids (i.e., primary metabolites) and the phenolics (i.e., secondary metabolites).

Immediately after removal from storage conditions and after additional 1 and 2 days in air at 20 °C, to simulate retail market conditions, fruit decay was visually evaluated. Any berries with visible shriveling and mold growth were considered decayed [18].

2.3. Sample Extraction

For the individual sugar and organic acid extractions [19], the freshly thawed blueberry fruit were finely chopped with a knife, and 1 g was mixed with 4 mL bi-distilled water in a test tube, as five repetitions. The samples were left at room temperature for 30 min for extraction of the sugars and organic acids, with constant agitation (Unimax 1010; Heidolph, Schwabach, Germany). This was followed by centrifugation at $9000\times g$ at 4 °C for 10 min (5810 R; Eppendorf, Hamburg, Germany). The samples were filtered through cellulose filters (Chromafil A-20/25; Macherey-Nagel, Düren, Germany) into vials, and stored at -20 °C until further analysis.

The samples for extraction of the phenolics were initially prepared as for the individual sugar and organic acid extractions, with five replicates per condition [20]. Here, 3 g finely chopped fruit was mixed with 5 mL extraction solution (70% methanol, 3% formic acid, in bi-distilled water) in test tubes. The samples were mixed by vortexing, and left in a cooled ultrasonic bath (0 °C) for 1 h. After this extraction of the phenolics from the plant tissue, the samples were centrifuged for 10 min at $9000\times g$ at 4 °C (5810 R; Eppendorf, Hamburg, Germany) and filtered through 0.2- μ m polyamide filters (Chromafil AO-20/25; Macherey-Nagel, Düren, Germany) into vials. These samples were stored at -20 °C until further analysis.

2.4. Analytical Methods

Separation of the individual carbohydrates was achieved using an HPLC system (Vanquish; Thermo Scientific, Waltham, MA, USA) connected to a refractive index detector (RI plus, RefractoMax520, Thermo Scientific, Waltham, MA, USA) [21]. The injection volume was 20 μ L, the flow rate was 0.6 mL/min, and the column (Rezex RCM-monosaccharide Ca+ 2%; 300 mm $\times$ 7.8 mm; Phenomenex, Torrance, CA, USA) was run at 65 °C. The mobile phase was bi-distilled water, with a total run time of 30 min. The individual carbohydrates were identified by comparisons of their retention times with corresponding external standards (i.e., sucrose, glucose, fructose), and were quantified according to standard curves. These are expressed as mg/g fresh weight (FW).

Analysis and identification of the organic acids were achieved using the same HPLC system (Vanquish; Thermo Scientific, Waltham, MA, USA), which was connected here to a UV detector with absorbance at 210 nm, according to the method previously described

by Mikulic-Petkovsek et al. [21]. The separation column (Rezex ROA-Organic acid H+ 8%; 150 mm × 7.8 mm; Phenomenex, Torrance, CA, USA) was run at 65 °C. The 20 µL samples were analyzed over 15 min at a flow rate of 0.6 mL/min. The mobile phase was 4 mM sulfuric acid in bi-distilled water. The individual organic acids were identified by comparisons of their retention times with external standards (i.e., citric, tartaric, malic, shikimic acid), and their contents were calculated from standard curves. They are expressed as mg/g FW.

The phenolics were analyzed using an HPLC system (Dionex UltiMate 3000; Thermo Scientific, Waltham, MA, USA) with a diode array detector at absorbances of 280 nm, 350 nm, and 530 nm. The injection volume of 20 µL was separated on a C18 column (Gemini; 150 × 4.6 mm; 3 µm; Phenomenex, Torrance, CA, USA), at 25 °C and a flow rate of 0.6 mL/min. The autosampler temperature was set to 10 °C. The mobile phases were 3% acetonitrile, 0.1% formic acid, in bi-distilled water (A; *v/v/v*), and 3% bi-distilled water, 0.1% formic acid, in acetonitrile (B; *v/v/v*). The gradient used for the analysis was as follows: 0–15 min, 5% B; 15–20 min, 5–20% B; 20–30 min, 20–30% B; 30–35 min, 30–90% B; 35–45 min, 90–100% B; 45–50 min, 100–5% B [20].

Identification of the phenolics was obtained and confirmed by comparisons of the retention times with external standards and using mass spectrometry analysis (LTQ XL; Thermo Scientific, Waltham, MA, USA), based on mass fragmentation patterns. The mass spectrometry was operated in negative or positive (for anthocyanins) ion modes, with electrospray ionization. The conditions of the analysis were: injection volume, 10 µL; flow rate, 0.6 mL/min; capillary temperature, 250 °C; sheath gas, 20 units; and auxiliary gas, 8 units. The source voltage was 4 kV, and the scanning was from *m/z* 115 to *m/z* 1600. The phenolics contents were calculated from standard curves, and are expressed in mg/kg FW.

2.5. Statistical Analysis

Statistical analysis was performed using R commander i386 4.0.3 [22]. Differences between the data were analyzed using one-way analysis of variance (ANOVA), with estimations using Duncan tests, and significant set at $p < 0.05$. Additionally, multivariate analysis was used for data visualization.

3. Results

3.1. Fruit Weight

The fruit weights during storage are presented in Figure 1, with the corresponding statistical analysis provided in Supplementary Materials Table S1. In all four of the atmospheres, the fruit lost significant weight from harvest (storage: 0 days) until the end of storage (62 days). For the air control, there were significant differences from the initial weight to all of the later storage times, although no further significant weight loss was seen after 30 days of storage (Supplementary Table S1). For the various CO₂ conditions, the weight losses during storage to 62 days were generally less pronounced. However, while the blueberry fruit stored under 5% CO₂ initially paralleled the air control (to 17 days of storage), there was then significantly greater weight loss for 44 days and 62 days of storage. Instead, with 15% and 25% CO₂, there was slower initial weight loss to 30 days, and then these paralleled the unchanged air control to 62 days. Therefore, for the final measures at 44 days and 62 days, the fruit stored under 15% CO₂ had significantly lower weights compared to the other storage conditions.

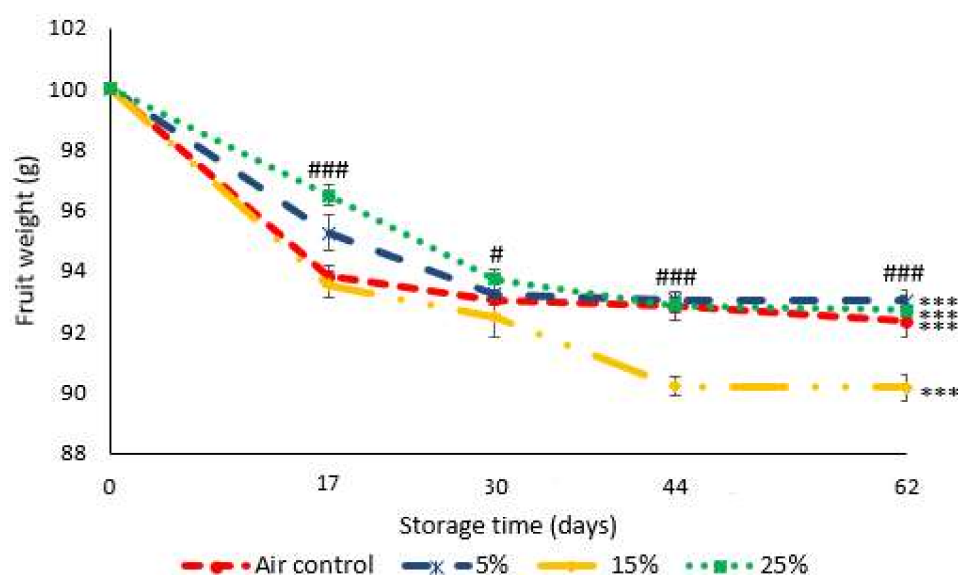


Figure 1. Blueberry ‘Liberty’ fruit weight loss under the air control and controlled atmospheres (5% CO₂, 5% O₂, 90% N₂ (5%); 15% CO₂, 5% O₂, 80% N₂ (15%); and 25% CO₂, 5% O₂, 70% N₂ (25%)) for different storage times. ***, $p < 0.001$ (versus storage = 0, within each storage condition). #, $p < 0.05$; ###, $p < 0.001$ (versus air storage, for each storage time).

Fruit decay control after removal from storage conditions and after 1 and 2 days on 20 °C showed no shriveling or mold growth.

3.2. Sugars

Figure 2 shows the variations in total sugars contents of the blueberry fruit under each storage conditions, with the corresponding statistical analysis provided in Supplementary Materials Table S1. As can be seen, the total sugars were influenced the most under the air control conditions, and the least with 25% CO₂. Within the first 17 days of storage, there were significant drops in the total sugars for all of the conditions, with the largest losses seen for the air control and 5% CO₂. However, after the initial losses over the first 17 days of storage, there were effectively no further changes in total sugars to 62 days under any of these storage conditions.

For the main individual sugars (Table 1), the sucrose content of the blueberry fruit (as 11% of total sugars) initially decreased significantly for the air control by 30 days of storage, and then remained essentially stable. In contrast, the main individual sugars in the blueberry fruit of glucose (36%) and fructose (53%) each showed significant breakdown for the air control in the first 17 days of storage, with no further loss to the final 62 days of storage. For 5% CO₂, the sucrose content significantly decreased over the first 30 days, and then remained unchanged (Table 1). The same was seen for glucose and fructose contents for 5% CO₂, with essentially little difference between the air control and 5% CO₂. Similar trends were seen for 15% CO₂, although the losses of each of the individual sugars were lower. The sucrose content during storage in 25% CO₂ remained essentially unchanged throughout the full 62 days of storage, with no significant difference between the start and end of storage. Both the glucose and fructose contents showed initial small, but significant, decreases over the first 17 days. The glucose contents then increased again to 30 days and remained unchanged to the end of storage. After the first 17 days of storage, the fructose contents remained unchanged.

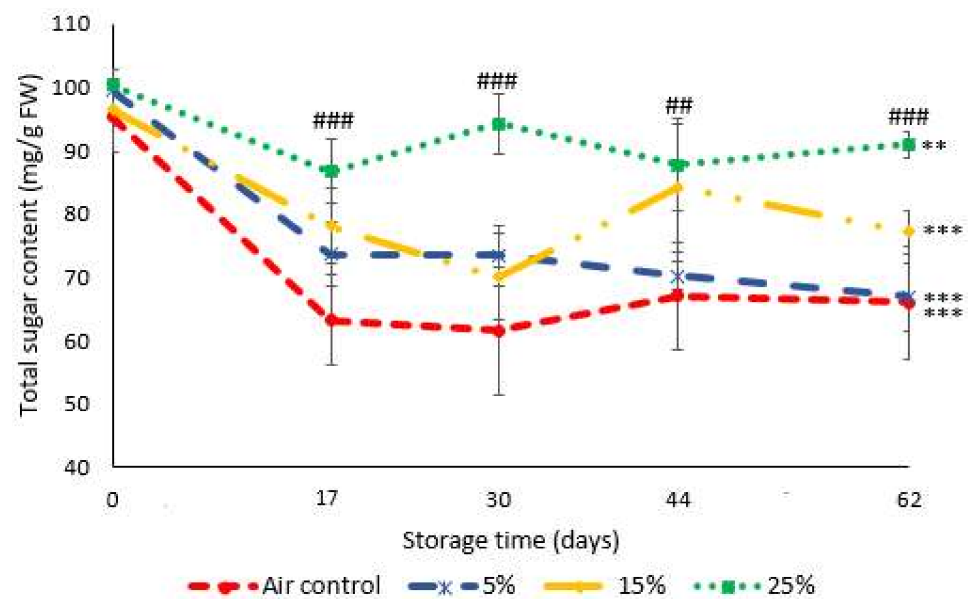


Figure 2. Blueberry ‘Liberty’ fruit total sugar content under the air control and controlled atmospheres (5% CO₂, 5% O₂, 90% N₂ (5%); 15% CO₂, 5% O₂, 80% N₂ (15%); and 25% CO₂, 5% O₂, 70% N₂ (25%)) for different storage times (mg/g FW). **, $p < 0.01$; ***, $p < 0.001$ (versus storage = 0, within each storage condition). ##, $p < 0.01$; ###, $p < 0.001$ (versus air storage, for each storage time).

Table 1. Contents of individual sugars of blueberry ‘Liberty’ fruit for the air control and controlled atmospheres of: 5% CO₂, 5% O₂, 90% N₂ (5% CO₂); 15% CO₂, 5% O₂, 80% N₂ (15% CO₂); and 25% CO₂, 5% O₂, 70% N₂ (25% CO₂), according to length of storage.

Condition	Storage Time (Days)	Sugar Content (mg/g FW)		
		Sucrose	Glucose	Fructose
Air control	0	10.56 ± 0.51 a	34.42 ± 2.06 a	50.34 ± 3.24 a
	17	8.81 ± 1.14 ab, B	22.24 ± 2.57 b, C	32.22 ± 3.81 b, D
	30	7.96 ± 1.11 b, C	21.61 ± 3.71 b, C	31.93 ± 6.03 b, C
	44	9.51 ± 1.30 ab	23.05 ± 3.14 b, B	34.47 ± 4.94 b, B
	62	9.65 ± 1.96 ab, B	22.68 ± 3.16 b, C	33.62 ± 4.75 b, C
	Significance	*	***	***
5% CO ₂	0	11.10 ± 0.38 a	35.76 ± 1.18 a	52.62 ± 1.81 a
	17	10.79 ± 1.43 ab, A	26.15 ± 1.68 b, B	36.67 ± 2.90 b, C
	30	8.73 ± 1.10 c, BC	26.08 ± 1.56 bc, B	38.63 ± 3.70 b, B
	44	9.89 ± 0.49 abc	24.47 ± 1.36 bc, B	35.80 ± 1.88 b, B
	62	9.27 ± 0.75 bc, B	23.08 ± 2.21 c, C	34.62 ± 2.50 b, C
	Significance	**	***	***
15% CO ₂	0	11.56 ± 0.11 a	34.35 ± 1.05 a	50.60 ± 1.75 a
	17	9.72 ± 0.66 ab, AB	27.34 ± 2.36 bc, B	41.04 ± 3.06 bc, B
	30	9.15 ± 0.72 b, B	24.07 ± 2.46 c, BC	36.85 ± 3.78 c, BC
	44	10.39 ± 1.58 ab	29.18 ± 3.73 b, A	44.58 ± 4.89 ab, A
	62	10.86 ± 1.49 ab, AB	26.45 ± 1.48 bc, B	39.83 ± 2.33 bc, B
	Significance	*	***	***
25% CO ₂	0	11.24 ± 0.29 ab	36.20 ± 0.30 a	52.90 ± 1.17 a
	17	10.67 ± 1.04 b, A	30.59 ± 2.30 c, A	45.45 ± 2.22 b, A
	30	12.90 ± 0.90 a, A	34.99 ± 1.63 ab, A	46.91 ± 2.36 b, A
	44	10.68 ± 0.64 b	32.23 ± 2.99 bc, A	44.86 ± 3.68 b, A
	62	11.88 ± 0.30 ab, A	33.85 ± 0.90 abc, A	45.20 ± 1.11 b, A
	Significance	**	**	***
Significance	17	*	***	***
	30	***	***	***
	44	NS	***	***
	62	*	***	***

Data are means ± standard errors (five replicates per condition). Different lowercase letters (a–c) indicate statistically significant differences between storage durations within each CO₂ condition; different uppercase letters (A–D) indicate statistically significant differences between CO₂ conditions within each storage duration (Duncan tests; $\alpha < 0.05$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

For all of the individual storage times from harvest (storage: 0 days) to 17, 30, 44, and 62 days of storage, the sucrose content tended to remain higher in the fruit with the higher CO₂ concentrations, with significance over the air control generally seen at both 30 days and 62 days of storage in 15% and 25% CO₂. These benefits of higher CO₂ were more accentuated for both glucose and fructose, and were seen for more or less all storage times from 17 days onwards; the highest glucose and fructose contents were for the fruit stored in atmospheres with 15% and 25% CO₂.

3.3. Organic Acids

From Figure 3 and Supplementary Materials Table S1, it can be seen that the total organic acids contents decreased the most for blueberry fruit stored under the air control condition. Together with 15% and 25% CO₂, these were conditions where there was significant decrease from harvest (storage: 0 days) to 17 days of storage. However, there were essentially no further changes in the total organic acids from the first 17 days until the end of storage under 15% and 25% CO₂, while they increased for the air control and decreased for 5% CO₂.

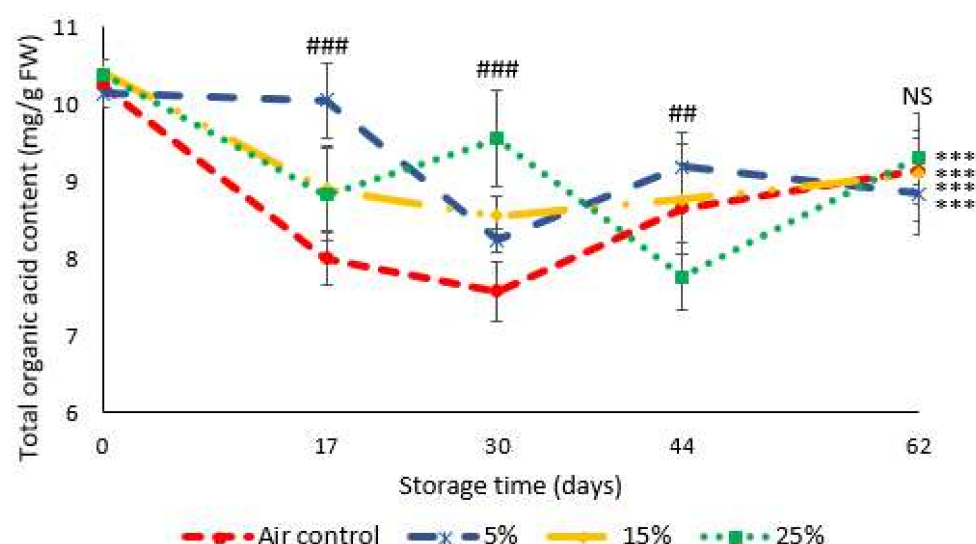


Figure 3. Blueberry ‘Liberty’ fruit total organic acid content under the air control and controlled atmospheres (5% CO₂, 5% O₂, 90% N₂ (5%); 15% CO₂, 5% O₂, 80% N₂ (15%); and 25% CO₂, 5% O₂, 70% N₂ (25%)) for different storage times (mg/g FW). ***, $p < 0.001$ (versus storage = 0, within each storage condition). ##, $p < 0.01$; ###, $p < 0.001$ (versus air storage, for each storage time).

The contents of individual organic acids in these blueberry fruit during storage are shown in Table 2. Among all the organic acids identified here, citric acid (90.1%) predominated, with only traces for tartaric acid (5.1%), malic acid (4.6%), and shikimic acid (0.2%). For the air control, citric and tartaric acids showed no significant decreases over the first 17 days of storage. All of the organic acids were significantly decreased by 30 days, with no further changes to the full 62 days of storage. Indeed, across the increasing CO₂ concentrations of each of the storage conditions (i.e., 5%, 15%, 25% CO₂), this pattern of changes remained across the individual organic acids. Thus, significant decreases generally occurred over the first 17 days and/or 30 days, followed by little or no further changes to 62 days.

Within the same storage durations, no particular trends were seen for the individual organic acids for the increasing CO₂ concentrations.

Table 2. Individual organic acids contents of blueberry ‘Liberty’ fruit for the air control and controlled atmospheres of: 5% CO₂, 5% O₂, 90% N₂ (5% CO₂); 15% CO₂, 5% O₂, 80% N₂ (15% CO₂); and 25% CO₂, 5% O₂, 70% N₂ (25% CO₂), according to length of storage.

Condition	Storage Time (Days)	Organic Acid Content (mg/g FW)			
		Citric	Tartaric	Malic	Shikimic
Air control	0	9.03 ± 0.10 a	0.51 ± 0.02 ab	0.67 ± 0.05 a	0.030 ± 0.004 a
	17	7.22 ± 1.00 ab, B	0.40 ± 0.07 bc, AB	0.36 ± 0.10 b	0.017 ± 0.001 b
	30	6.79 ± 0.99 b, B	0.38 ± 0.06 c, B	0.38 ± 0.03 b	0.015 ± 0.001 b, B
	44	7.88 ± 1.09 ab	0.43 ± 0.06 bc	0.32 ± 0.03 b, AB	0.016 ± 0.002 b, AB
	62	8.13 ± 1.53 ab	0.56 ± 0.10 a	0.43 ± 0.05 b, A	0.014 ± 0.001 b, AB
	Significance	*	**	***	***
5% CO ₂	0	8.90 ± 0.10 ab	0.55 ± 0.07	0.66 ± 0.53 a	0.024 ± 0.000 a
	17	9.11 ± 1.38 a, A	0.50 ± 0.10 A	0.42 ± 0.11 b	0.019 ± 0.002 b
	30	7.43 ± 1.03 b, B	0.44 ± 0.06 AB	0.35 ± 0.01 b	0.016 ± 0.001 c, B
	44	8.28 ± 0.44 ab	0.46 ± 0.04	0.43 ± 0.03 b, A	0.018 ± 0.001 bc, A
	62	7.91 ± 0.77 ab	0.48 ± 0.03	0.42 ± 0.05 b, A	0.016 ± 0.002 c, A
	Significance	*	NS	***	***
15% CO ₂	0	9.13 ± 0.13	0.57 ± 0.03 a	0.70 ± 0.04 a	0.023 ± 0.000 a
	17	8.23 ± 0.51 AB	0.34 ± 0.04 b, B	0.31 ± 0.04 b	0.019 ± 0.001 b
	30	7.75 ± 0.71 AB	0.43 ± 0.04 ab, AB	0.36 ± 0.05 b	0.016 ± 0.002 bc, AB
	44	7.86 ± 1.51	0.48 ± 0.15 a	0.40 ± 0.16 b, A	0.015 ± 0.002 c, AB
	62	8.27 ± 1.26	0.49 ± 0.06 a	0.33 ± 0.09 b, B	0.013 ± 0.002 c, B
	Significance	NS	*	***	***
25% CO ₂	0	9.11 ± 0.07 a	0.54 ± 0.03 a	0.71 ± 0.02 a	0.03 ± 0.000 a
	17	8.04 ± 0.49 b, AB	0.43 ± 0.07 bc, AB	0.34 ± 0.05 b	0.018 ± 0.000 b
	30	8.72 ± 0.55 ab, A	0.48 ± 0.06 ab, A	0.34 ± 0.05 b	0.019 ± 0.002 b, A
	44	7.12 ± 0.69 c	0.37 ± 0.06 c	0.26 ± 0.04 c, B	0.015 ± 0.001 c, B
	62	8.52 ± 0.32 ab	0.50 ± 0.04 ab	0.28 ± 0.02 bc, B	0.014 ± 0.001 c, AB
	Significance	***	**	***	***
Significance	17	*	*	NS	NS
	30	*	.	NS	*
	44	NS	NS	*	*
	62	NS	NS	**	.

Data are means ± standard errors (five replicates per condition). Different lowercase letters (a–c) indicate statistically significant differences between storage durations within each CO₂ condition; different uppercase letters (A–B) indicate statistically significant differences between CO₂ conditions within each storage duration (Duncan tests; $\alpha < 0.05$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.4. Sugars to Organic Acids Ratio

From harvest (storage: 0 days) to the first sampling date at 17 days, small but significant decreases were seen for the total sugars-to-organic acids ratio for fruit stored in the air control and with 5% CO₂ (Figure 4), which provided some of the lowest sugars-to-organic acids ratios for this fruit (Supplementary Material Table S1). In contrast, significant increases in the sugars-to-organic acids ratio were seen for fruit stored in 15% and 25% CO₂, although only towards the end of storage (i.e., at 44 days). Furthermore, for 25% CO₂, this effect was greater, as the sugars-to-organic acids ratio was consistently increased throughout storage, defining the highest sugars-to-organic acids ratios. In the last storage samples, at 62 days, the sugars-to-organic acids ratio for 25% CO₂ then showed a significant drop, with no significant difference compared to the increase seen for 15% CO₂.

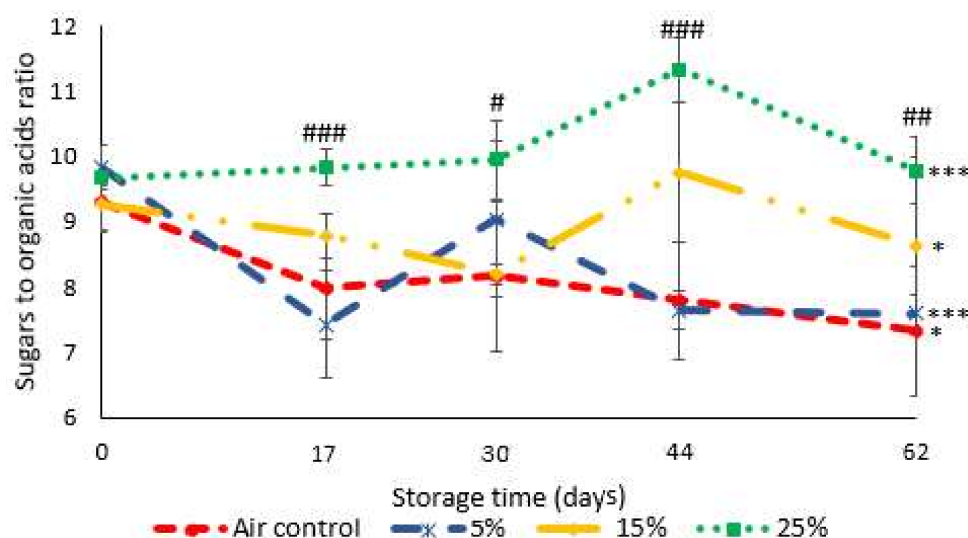


Figure 4. Blueberry ‘Liberty’ fruit sugars-to-organic acid ratio under the air control and controlled atmospheres (5% CO₂, 5% O₂, 90% N₂ (5%); 15% CO₂, 5% O₂, 80% N₂ (15%); and 25% CO₂, 5% O₂, 70% N₂ (25%)) for different storage times. *, $p < 0.05$; ***, $p < 0.001$ (versus storage = 0, within each storage condition). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ (versus air storage, for each storage time).

3.5. Individual Phenolics

Figure 5 shows a heatmap for the individual phenolics contents, and Supplementary Material Table S2 groups the phenolics contents in these blueberry fruit according to duration of storage within the air control and increasing CO₂ concentrations. As can be seen in general, the greatest reductions relative to the start of storage (i.e., Figure 5, changes from white to red) occurred within the first 17 days of storage for 15% and 25% CO₂. Here, in the air control, among the flavan-3-ols, the epicatechin content decreased the most from the start to 17 days of storage (by 30%), while the other flavan-3-ols showed smaller losses. Among the hydroxycinnamic acids, the ferulic acid derivative varied during storage, but then reached its lowest at 62 days (for 30% reduction), while 5-caffeoylquinic acid constantly increased through storage to 44 days (by 50%). In the flavonol group, still in the air control, the contents of all of these compounds varied widely during storage, as also for the individual anthocyanins; however, over the last 17 days of storage, all of the anthocyanins showed some decreases.

In the storage with 5% CO₂, all of the flavan-3-ols initially showed reductions (i.e., to 17 days of storage), with the most pronounced for epicatechin content (by 35%). The ferulic acid derivative and feruloyl glucose contents were generally stable throughout storage, while 4-caffeoylquinic acid decreased. The flavonol content remained relatively high throughout storage, with myricetin-3-O-rhamnoside, quercetin-3-O-arabinofuranoside, and isorhamnetin-3-O-glucuronide in particular showing reductions for longer sampling times. There was relatively low anthocyanin breakdown for 5% CO₂, with cyaniding-3-O-arabinoside, petunidin-3-O-arabinoside, peonidin-3-O-galactoside, and peonidin pentose, in particular, showing reductions in the second half of the storage period (i.e., 30–62 days).

However, for 15% and 25% CO₂, there were notable reductions for all of the phenolics in general (i.e., Figure 5, predominance of red). However, for the flavan-3-ols, epicatechin tended to increase in the second half of storage. Moreover, against the trend, among the hydroxycinnamic acids, 4-caffeoylquinic acid content showed a recovery with longer storage, while the other phenolics in this group generally decreased. For the various flavonol syringetin, quercetin, kaempferol, and isorhamnetin derivatives identified, their contents generally initially decreased in 15% CO₂, while from 30 days onwards, the contents of all of the flavonols remained steady, or even slightly increased. For the flavonols and anthocyanins for 25% CO₂, their contents all generally decreased throughout storage to 62 days, with the greatest reduction for myricetin-3-O-hexoside. However, in 15% CO₂, the

anthocyanin contents showed their major decrease to 30 days of storage, with most then recovering somewhat by end of storage.

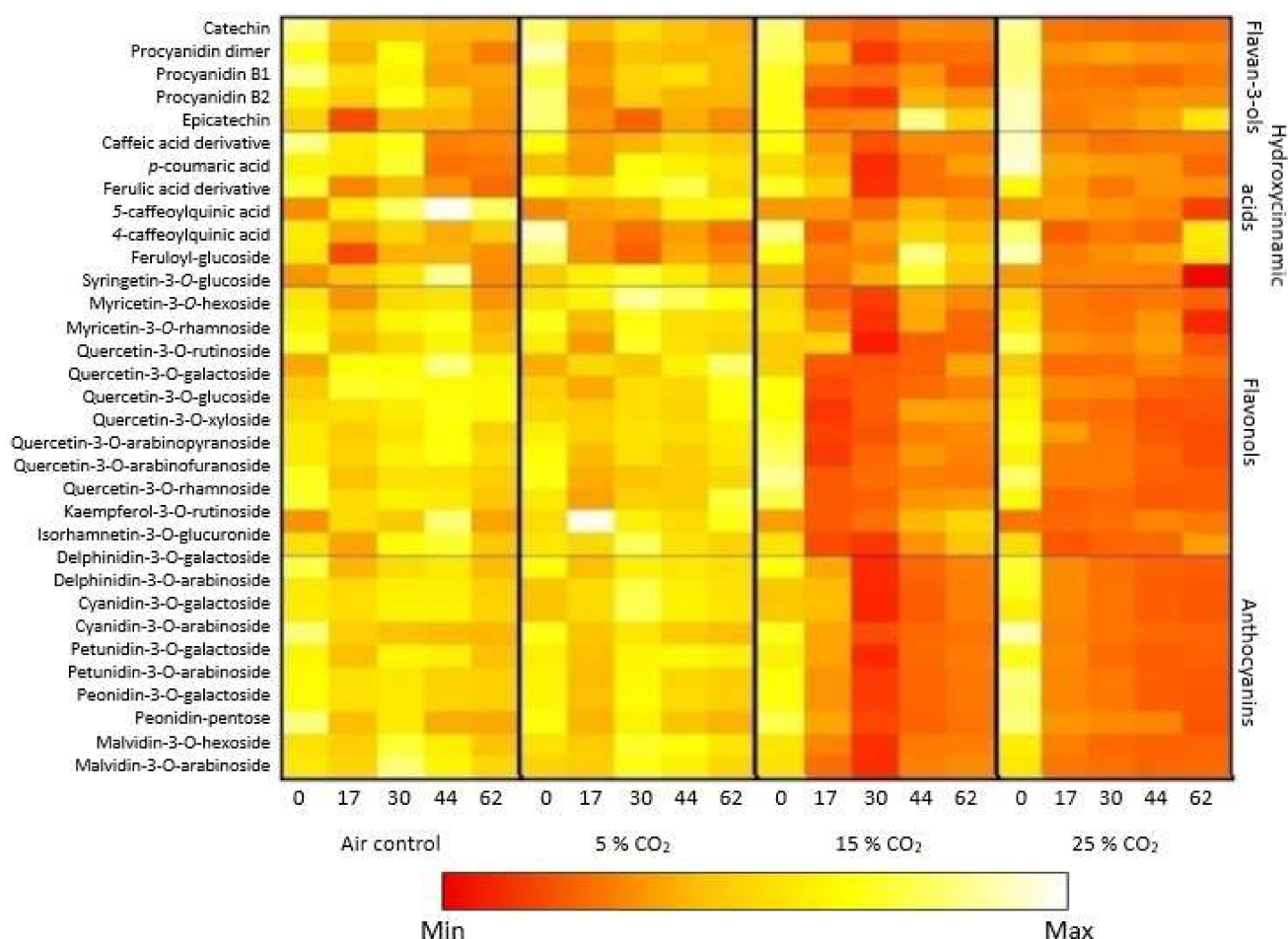


Figure 5. Heatmap for blueberry ‘Liberty’ fruit individual phenolics contents under the air control and controlled atmospheres (5% CO₂, 5% O₂, 90% N₂ (5%); 15% CO₂, 5% O₂, 80% N₂ (15%); and 25% CO₂, 5% O₂, 70% N₂ (25%)) for different storage times. Red, low individual phenolics content; white, high individual phenolics content.

4. Discussion

After harvest, there are increases in physiological and chemical processes that accelerate senescence and alter the quality of these blueberry fruit. These processes are mainly connected to respiration, which provides the fruit with energy and organic molecules that protect the fruit cells. The respiration rate and shelf-life of the fruit show an inverse relationship, which means that with greater respiration, there is faster fruit deterioration [23]. At present, lower respiration rates after harvesting of blueberry fruit are achieved by cold storage and modifications to their atmosphere during storage. The latter here is effective because lower O₂ levels (i.e., generally between 2% and 3%) slow down respiratory rates and ethylene production [23,24]; however, most fruits possess low O₂ threshold (approximately 1%), below which fermentation is likely to occur [15,23]. Together with a low O₂ concentration, an appropriate CO₂ concentration should be established, due to their strong interactions on the performance of blueberry fruit under modified storage conditions. The optimal O₂/CO₂ combination differs between different cultivars [15].

The weight of blueberry fruit is mainly lost through transpiration, which increases as the temperature of the atmosphere rises [25]. In the present study, the blueberry fruit were stored at 1 °C, so in general the temperature will not have affected the fruit weight loss either for the air control or for controlled atmosphere conditions. Weight loss that occurred

in the study did not result from fruit deterioration, since no fruit decay was observed after storage. In the air control, the significant decrease in fruit weight from 17 days to 30 days of storage is in agreement with maintenance of the quality parameters of blueberry fruit for 18 days under standard atmosphere storage conditions [13]. The differences in weight loss at 30 days of storage were significant between these CO₂ conditions; however, these small differences are negligible in practice. The significantly greater weight loss for 15% CO₂ over the second half of storage will be due to this prolonged exposure of the fruit to high CO₂ concentrations, which can lead to cellular damage, with a consequently greater weight loss [26]. At the same time, Duarte et al. [16] stated that higher weight loss results from over ripening of fruit can be associated with higher CO₂ concentrations. The relatively constant weight loss of the fruit in 5% CO₂ from 30 days of storage, combined with the greater weight losses in 15% CO₂ confirm that the optimal CO₂ concentration for the control of fruit decay is low, and is usually close to the tolerance level [27]. CO₂ concentrations above the fruit CO₂ threshold can lead to fermentation or toxicity [8]. Fruit damage is also likely to occur with high CO₂ [28], which is confirmed in the present study. Falagán et al. [29] reported that fruit weight loss differed between a standard air atmosphere and their controlled atmosphere conditions (i.e., sudden exposure to 5 kPa O₂ and 10 kPa CO₂, and gradually reaching the same O₂ and CO₂ concentrations in 3 and 7 days), with their controlled atmosphere conditions significantly slowing the rate.

Zheng et al. [18] reported that modified atmospheres with higher O₂ levels (60–100% O₂, balanced with N₂) reduced fruit decay in ‘Duke’ fruit. In our study, no mold growth or fruit shriveling was observed in fruit from all of the modified atmospheres where only 5% O₂ was maintained. In controlled atmosphere storage, a high O₂ atmosphere (i.e., above 70%) can be used as an alternative to low O₂ when in combination with a high CO₂ concentration because, as already mentioned, the higher CO₂ concentration that is required for fruit decay control is usually close to the tolerance level of the fruit [27]. Although, the mechanisms by which high O₂ atmospheres inhibit fruit decay are still unclear, it is possible that O₂ concentrations above 40% has toxic effect on microbial growth [18]. Higher fruit decay in the study of Zheng et al. [18] might have also been a consequence of their higher storage temperature (5 °C) compared to ours (0 °C). In addition, they did not include storage with different CO₂ concentrations. As already indicated, in modified atmosphere storage, the optimal O₂ concentration together with higher and appropriate CO₂ levels for the reduction of fruit decay is 2% to 3% [23]. In terms of N₂, 0% to 60% N₂ was used by Zheng et al. [18], while 70% to 90% N₂ was added in the present study. It is not clear whether the difference in N₂ will have any impact on fruit decay and shelf life, as this has yet to be studied.

According to Alsmairat et al. [15], fruit decay is cultivar dependent. Based on their results, ‘Liberty’ fruit showed the lowest mold growth and fruit decay among all of their cultivars in all atmospheres after 8 weeks of storage at 0 °C, which agrees with our findings. At the same time, they showed that an atmosphere with higher CO₂ together with lower O₂ concentrations suppressed mold growth to the greatest extent, thus leading to the lowest fruit decay. As the ratio of CO₂ to O₂ increased, the level of fruit decay decreased [15].

It is generally known that sugars and organic acids are the main substrates in respiratory metabolism. Combined with other postharvest factors, respiratory metabolism is also influenced by the gas composition of the atmosphere around the fruit [17]. Optimal storage temperatures differ between different fruit species, and respiration rates can be reduced by lower O₂ or higher CO₂ concentrations [23]. This can result in changes in the levels of individual sugars, as was also seen in the present study. The glucose and fructose contents significantly decreased from harvest (i.e., storage: 0 days) to the end of storage (i.e., 62 days) under all four of the conditions here (with the exception of glucose for 25% CO₂); however, with increasing CO₂ concentrations, the differences between the start and end of storage were lower. The reduction in sugar degradation with increased CO₂ concentration is also shown by the significantly higher individual and total sugar contents in the blueberry fruit stored in 25% CO₂ at all storage times. On the contrary, the sucrose contents during

storage suggested that sucrose served as a substrate for respiration over the first 30 days of storage, following which, the sucrose content no longer decreased. Contradictory data on sucrose and glucose contents of blueberry fruit after harvest were reported by Falagán et al. [29], where these sugars decreased and increased during storage under their control and controlled atmosphere conditions, respectively. Catuneanu et al. [17] showed significant increases in total soluble solids content in the blueberry fruit 'Chandler' under their control and 5% and 10% CO₂ controlled atmosphere conditions. From the present study, we can conclude that higher CO₂ concentrations can maintain higher total sugars content. Of the main sugars we identified, sucrose had the lowest content, and therefore contributed the least to the total sugar content.

According to Saltveit [23], increased CO₂ concentrations can stimulate fermentation. The citric and tartaric acid contents remained unchanged throughout the storage period under all of our storage conditions. Instead, malic and shikimic acids showed decreases throughout storage in these air control and controlled atmosphere conditions. This is contrary to Falagán et al. [29], where the citric acid content of the blueberry fruit 'Duke' decreased in their control, while it was maintained during storage under their controlled atmosphere conditions. Duarte et al. [16] measured higher titratable acidity in the blueberry fruit 'Brigitta' during their controlled atmosphere storage. As for the sugars, elevated CO₂ concentrations prevented the total organic acids breakdown that occurs during storage; however, after 30 days of storage, this was no longer the case.

The total sugars-to-organic acids ratio contributes to the flavor of blueberry fruit [30]. High sugar or low organic acid contents result in a sweet taste for the fruit, which means that the higher the sugars-to-organic acids ratio, the sweeter the fruit will taste [31]. In the fruit under 25% CO₂, the sugars-to-organic acids ratio increased during storage, which will be a result of lower sugar degradation, and the general weight loss due to water loss during transpiration [16].

Among various factors, fruit quality is also determined by the phenolics content, which in non-climacteric fruit, such as blueberry, is highest at harvest [23]. This is in agreement with the present study, where the majority of the individual phenolics decreased throughout storage, most prominently with 15% and 25% CO₂. This indicates that the blueberry fruit 'Liberty' has a low CO₂ threshold, which according to the present study, is 5%, above which concentration there were negative effects on the phenolics contents. For strawberry, the fruit sensitivity to CO₂ is both cultivar and species dependent [28]. Here, for blueberry, the individual phenolics contents during storage were maintained, and sometimes even increased, such as for 5-caffeoylquinic acid and the quercetin derivatives for the air control, and syringetin-3-O-glucoside and kaempferol-3-O-rutinoside for 5% CO₂. These effects will probably be a result of water loss in the fruit [16]. Alternatively, the flavan-3-ols and hydroxycinnamic acids showed considerable reductions under all of these conditions, which indicates poor storage stability of these compounds. On the other hand, the flavonols and anthocyanins showed a decreasing trend at 15% and 25% CO₂, while there were no consistent trends under the air control and 5% CO₂ conditions throughout the storage period. We also saw an increase for delphinidin and malvidin derivatives, and for petunidin-3-O-galactoside, over the first 17 days of storage in 5% CO₂, which is partly in agreement with Duarte et al. [16], who showed an increase in total anthocyanin content for up to 24 days of storage under 5%, 10%, and 15% CO₂. As the color of blueberry fruit depends on the anthocyanins content, the color of the fruit was not altered in the air control and with 5% CO₂, while this particularly important parameter (in terms of consumer perception of fruit quality and visual attraction) deteriorated in 15% and 25% CO₂. Opposite results were reported by Catuneanu et al. [17] for the blueberry fruit 'Blueray', where total anthocyanins content decreased from harvest to 8 weeks of storage in their control, and increased under 5% CO₂ storage.

5. Conclusions

In the present study, storage of blueberry fruit was evaluated under four different controlled atmosphere conditions over 62 days. This study shows that storage of these fruit significantly reduces their weight and phenolics contents, to different extents across the applied conditions. Weight loss and variations in the phenolics during storage were minimal for 5% CO₂, from which we can conclude that this storage atmosphere was optimal to preserve maximal fruit weight and quality of the blueberry ‘Liberty’ for up to 62 days, from the selected location. To maintain the total sugars-to-organic acids ratio and the phenolics contents at high levels, the fruit included in our experiment could be stored for up to 44 days under a standard air atmosphere, which can be increased to 62 days in an atmosphere with 5% CO₂. For preservation of the individual phenolics, the two higher CO₂ conditions here (i.e., 15%, 25% CO₂) are not recommended as controlled atmosphere storage conditions for the blueberry ‘Liberty’ fruit.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/horticulturae7110478/s1>, Table S1: Weight and total sugars and organic acids of blueberry fruit for the air control and the controlled atmospheres of 5% CO₂, 5% O₂, 90% N₂ (5% CO₂), 15% CO₂, 5% O₂, 80% N₂ (15% CO₂), and 25% CO₂, 5% O₂, 70% N₂ (25% CO₂), according to length of storage. Table S2: Hydroxycinnamic acids, flavan-3-ols, flavonols, anthocyanins and total phenolics of blueberry fruit for the air control and the controlled atmospheres of 5% CO₂, 5% O₂, 90% N₂ (5% CO₂), 15% CO₂, 5% O₂, 80% N₂ (15% CO₂), and 25% CO₂, 5% O₂, 70% N₂ (25% CO₂), according to length of storage.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Conflicts of Interest: The authors declare no conflict of interest.

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