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Carbon Dioxide–induced Changes in Color and Anthocyanin Synthesis of Stored Strawberry Fruit

Deirdre M. Holcroft[1] and Adel A. Kader[2]
Department of Pomology, University of California, One Shields Avenue, Davis, CA 95616

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Abstract. **Anthocyanin concentrations increased in both external and internal tissues of 'Selva' strawberries (*Fragaria ×ananassa* Duch.) stored in air at 5 °C for 10 days, but the increase was lower in fruit stored in air enriched with 10 or 20 kPa CO_2. Flesh red color was less intense in CO_2 storage than in air storage. Activities of phenylalanine ammonia lyase (PAL) and UDP glucose : flavonoid glucosyltransferase (GT) decreased during storage, with decreases being greater in both external and internal tissues of strawberry fruit stored in air + 20 kPa CO_2 than in those kept in air. Activities of both PAL and GT in external tissues of strawberries stored in air + 10 kPa CO_2 were similar to those in fruit stored in air, while enzyme activities in internal tissues more closely resembled those from fruit stored in air + 20 kPa CO_2. Phenolic compounds increased during storage but were not affected by the storage atmosphere. The pH increased and titratable acidity decreased during storage; these effects were enhanced in internal tissues by the CO_2 treatments, and may in turn have influenced anthocyanin expression.**

Anthocyanin synthesis continues in harvested fruit, particularly those stored in air, even at low storage temperatures. Increases have been observed in lowbush blueberry fruit (*Vaccinium angustifolium* Ait.) (Kalt and McDonald, 1996), where anthocyanin concentration increased by 18% after storage for 2 weeks at 1 °C; in pomegranates (*Punica granatum* L.), where an increase of 71% was observed after 6 weeks of storage at 10 °C (Holcroft et al., 1998); and in 'Blomidon' (Kalt et al., 1993) and 'Selva' strawberry fruit (19% increase in whole fruit or 31% in external tissues after storage for 10 d at 5 °C) (Gil et al., 1997). Treatment with CO_2 inhibits this postharvest increase in anthocyanin concentration (Gil et al., 1997; Holcroft et al., 1998) by affecting anthocyanin biosynthesis, degradation, or both.

The biosynthesis of anthocyanins has been well studied (Holton and Cornish, 1995; Stafford, 1990). The first step of the phenylpropanoid pathway is the production of cinnamic acid from phenylalanine, produced via the shikimic acid pathway, by the action of phenylalanine ammonia lyase (PAL; EC 4.3.1.5). Cinnamic acid is converted into coumaric acid, which is modified to the CoA form. Three molecules of malonyl CoA combine with ρ-coumaroyl-CoA and form naringenin chalcone, which is then converted into the flavanone, naringenin. The next step is the formation of dihydroflavonol, which is reduced to flavan-3,4-diol, or leucoanthocyanin, and then is converted to anthocyanidin. Finally, the glucose molecule is attached by the action of UDP glucose : flavonoid glucosyltransferase (GT; EC 2.4.1.91), resulting in the anthocyanin.

Our hypothesis is that elevated CO_2 atmospheres during storage adversely affect anthocyanin biosynthesis in strawberry fruit, and that this effect is greater in the internal tissues of the fruit. To test this hypothesis we measured anthocyanin concentration and the specific activity of PAL and GT, two important enzymes involved in biosynthesis, in external and internal tissues of strawberry fruit. Other phenolic compounds were also analyzed in an attempt to understand whether the storage atmosphere could affect the partitioning of the precursors into various phenolic compounds. Since color expression and stability of anthocyanins is affected by pH, we report CO_2-induced pH changes in fruit tissues.

Materials and Methods

Experimental setup. 'Selva' strawberries were harvested in Watsonville, Calif., and transported to the Univ. of California, Davis, in an air-conditioned vehicle and stored overnight at 5 °C. Damaged berries were discarded and remaining berries were sorted for uniform size and color. Sixty fruit were placed in each 9.5-L jar and three jars (replicates) were used per treatment. The jars were ventilated with a continuous flow of humidified air, air + 10 or air + 20 kPa CO_2 at a flow rate of 170 $mL \cdot min^{-1}$ using needle valve flow boards. The gases were maintained within 10% of the required concentration (e.g., 10 ± 1 kPa). The composition of the gases was checked regularly with a gas chromatograph equipped with a thermal conductivity detector (model 211; Carle Instruments, Anaheim, Calif.) or an infrared gas analyzer (model 2000R; Horiba Instruments, Irvine, Calif.).

Color. All the fruit were analyzed initially and after 5 and 10 d of storage at 5 °C. At each evaluation time 12 fruit per replicate were returned to the jars and held in air at 5 °C for a further 3 d. External color was measured on opposite sides of the fruit using a Minolta chromameter (model CR-200; Minolta, Ramsey, N.J.), which provided CIE L*, a*, and b* values. These values were used to calculate chroma ($C^* = [a^{*2} + b^{*2}]^{1/2}$), which indicates the intensity or color saturation, and hue angle (h° = arctangent [b*/a*]) where 0° = red-purple; 90° = yellow; 180° = bluish-green and 270° = blue (McGuire, 1992). The fruit were sliced in half and internal flesh color was measured on both halves. External and internal color was also measured on the fruit that had been returned to air for 3 d.

pH, TA, and TSS. Fruit halves were separated into external tissues (epidermis and about one-third of the cortex), internal (pith, bundle zone, and the remainder of the cortex), and the juice was analyzed separately for total soluble solids (TSS), pH, and titratable acidity (TA). Soluble solids content was measured using an Abbe refractometer (model 10450; American Optical, Buffalo, N.Y.). A 4-g sample of juice was diluted with 20 mL of distilled water, and pH and TA were measured using an automatic titrator fitted with pH meter and an autoburette (PHM85 Precision, ABU80; Radiometer, Copenhagen, Denmark). TA was expressed as percentage of citric acid.

Anthocyanin extraction and analysis. Twenty halves per replicate were frozen in liquid N_2 and kept at –80 °C until analyzed for anthocyanin and enzyme activities. The frozen tissue was removed from the freezer, allowed to thaw slightly, and separated into external and internal tissues as described previously. The separated tissue was immediately refrozen in liquid N_2 before being ground in an Oster blender. Anthocyanins were extracted, separated, and quantified as described by Gil et al. (1997).

Enzyme extraction and analysis. The method of Lister et al. (1996) was used to extract and analyze PAL and GT, with the only difference being in the GT assay. UDP-glucose replaced UDP-galactose, and the product, quercetin 3-glucose, was quantified by the high-performance liquid chromatography (HPLC) method described in Holcroft et al. (1998).

Total and individual phenolics. Total soluble phenolic concentration was measured with a commercially available Folin & Ciocalteau phenol reagent (Sigma Chemical Co., St. Louis) using ρ-coumaric acid as a standard (Singleton and Rossi, 1965). The extracts were prepared for HPLC analysis by dilution with 80% ethanol prior to analysis. All samples and standards were run in duplicate and absorbance was measured at 660 nm.

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[1]Current address: Dept. of Horticultural Science, Univ. of Stellenbosch, P. Bag X1, Matieland 7602, South Africa.
[2]To whom reprint requests should be addressed.

The samples prepared for anthocyanin analysis were analyzed for phenolics by HPLC using the method described in Gil et al. (1997).

Total protein. Total protein was extracted and measured from 1 g tissue initially and after 10 d of storage (Rothan et al., 1997).

Statistical analysis. Analysis of variance (ANOVA) of the main effects and LSD values with a significance level of 5% were obtained using CoStat Statistical Software (version 5.01; CoHort Software, Minneapolis). Presented data points are the means of three replications ±SE.

Results and Discussion

Skin and flesh color. Skin (external) color of strawberries became slightly darker (L* decreased) with time in storage but was not affected by storage atmosphere (Fig. 1A). The chroma values increased with time, and this increase was greater after 5 d storage in CO_2 than in air. After 10 d at 5 °C chroma was not affected by storage atmospheres (Fig. 1C). However, the hue angle of berries held in air was lower than that of those held in either CO_2 treatment (Fig. 1E).

The flesh (internal) color showed the most interesting changes in relation to the treatments, and L*, chroma, and hue were significantly affected ($P \leq 0.05$) by CO_2 treatments; L* was lower (darker) (Fig. 1B) and the chroma was higher in air-stored fruit after 10 d (Fig. 1D). The chroma value of the fruit stored in air + 10 kPa CO_2 was slightly higher than that of those stored in air + 20 kPa CO_2. These results confirm previous observations (Gil et al., 1997); CO_2 treatments maintained a lighter color of the internal tissue of 'Selva' strawberry during storage, although the effect was not as marked in this experiment. The hue angle increased most, i.e., became more orange red, after storage in air + 10 kPa CO_2 (43.8 after 10 d) (Fig. 1F). This agrees with our previous data where air + 10 kPa CO_2 also had a greater effect on hue angle (51.3 after 10 d) (Gil et al., 1997).

The 12 fruit from each replicate that was transferred to air at 5 °C for a further 3 d, after 5 and 10 d in air + 10 or air + 20 kPa CO_2, showed no increase in red color (data not shown).

Anthocyanin concentration. The total anthocyanin concentration in external tissue increased during storage in all treatments ($P \leq 0.05$) (Fig. 2A). The increase (146 $\mu g \cdot g^{-1}$) was greatest in air-stored fruit, reaching a maximum of 530 $\mu g \cdot g^{-1}$ after 10 d. Fruit stored in air did not differ significantly from those stored in 10 kPa CO_2-enriched air. The increase in anthocyanin concentration in fruit stored in air + 20 kPa CO_2 was 47 $\mu g \cdot g^{-1}$ over the 10-d storage period. The anthocyanin concentration in fruit stored in air + 20 kPa CO_2 was significantly ($P \leq 0.05$) lower than in fruit in the other two treatments after 10 d.

Initially, the anthocyanin concentration in the internal fruit tissues was much lower than in the external tissues (118 vs. 384 $\mu g \cdot g^{-1}$, respectively). An increase of 38 $\mu g \cdot g^{-1}$ of anthocyanin was measured in internal tissue of air-stored fruit over the 10-d period, while no increase occurred in fruit stored in air + 20 kPa CO_2 (Fig. 2B). The major difference between external and internal tissues was in the response of fruit stored in air + 10 kPa CO_2; anthocyanin concentration in the external tissue was similar to that of air-stored fruit, while that in the internal tissue was similar to that of fruit stored in air + 20 kPa CO_2.

The anthocyanin concentrations appear higher than our previously reported concentrations (Gil et al., 1997), but in the first instance we used a standard of cyanidin 3-glucoside (Cy 3-G) to quantify the anthocyanin concentration and in this work we used pelargonidin 3-glucoside (Pg 3-G). A comparison of peak areas from the chromatograms indicates that the concentrations were similar in the two experiments; Pg 3-G was the most abundant pigment in strawberry fruit, and accounted for ≈88% of the anthocyanin in external tissues and 96% in internal tissues, while pelargonidin 3-rutinoside (Pg 3-R) accounted for 4% of the total anthocyanin in both exter-

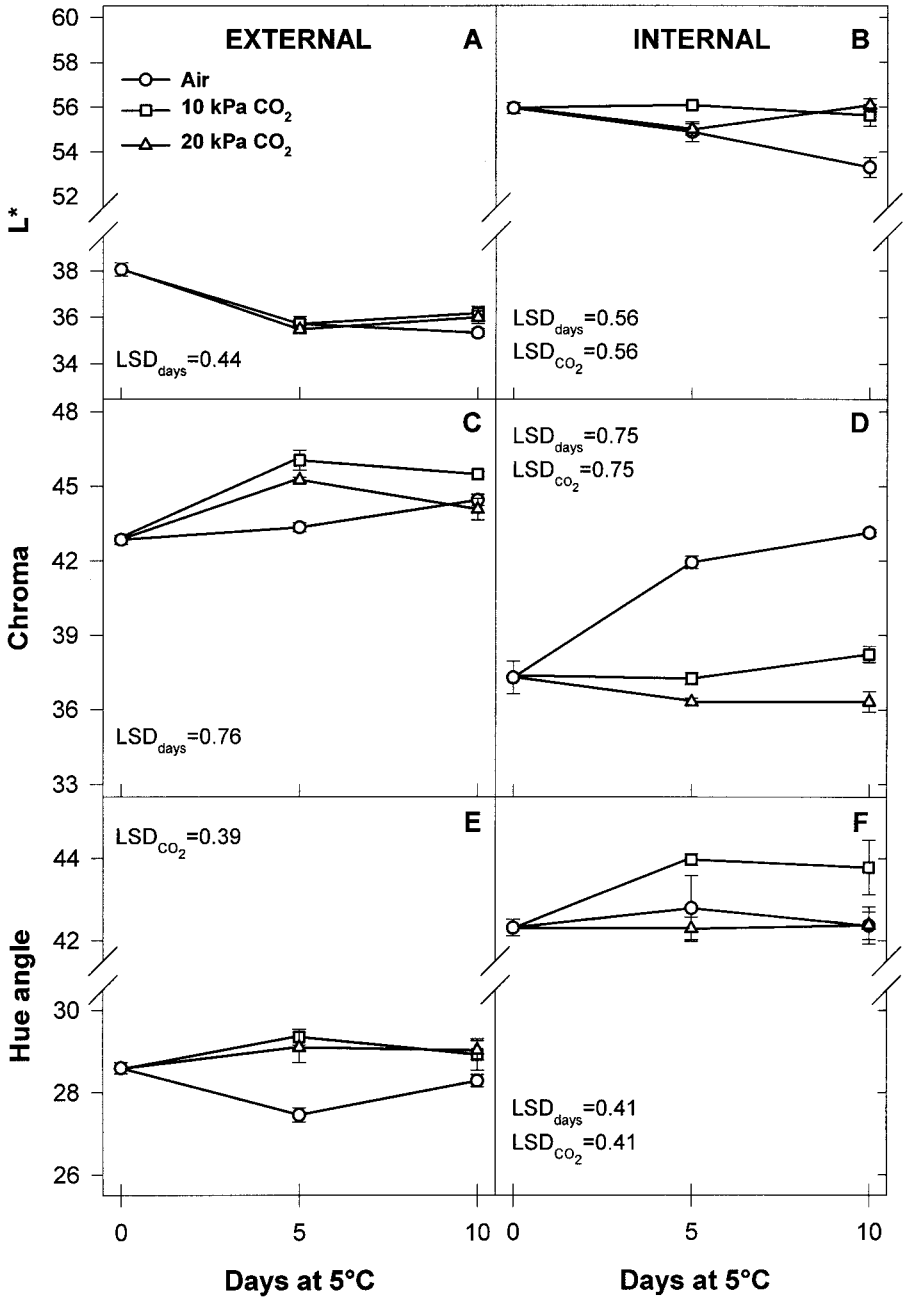

Fig. 1. External (skin) and internal (flesh) color, measured as L* or lightness (**A, B**), chroma (**C, D**) and hue angle (**E, F**), of 'Selva' strawberries stored at 5 °C for 5 or 10 d in air or CO_2-enriched atmospheres.

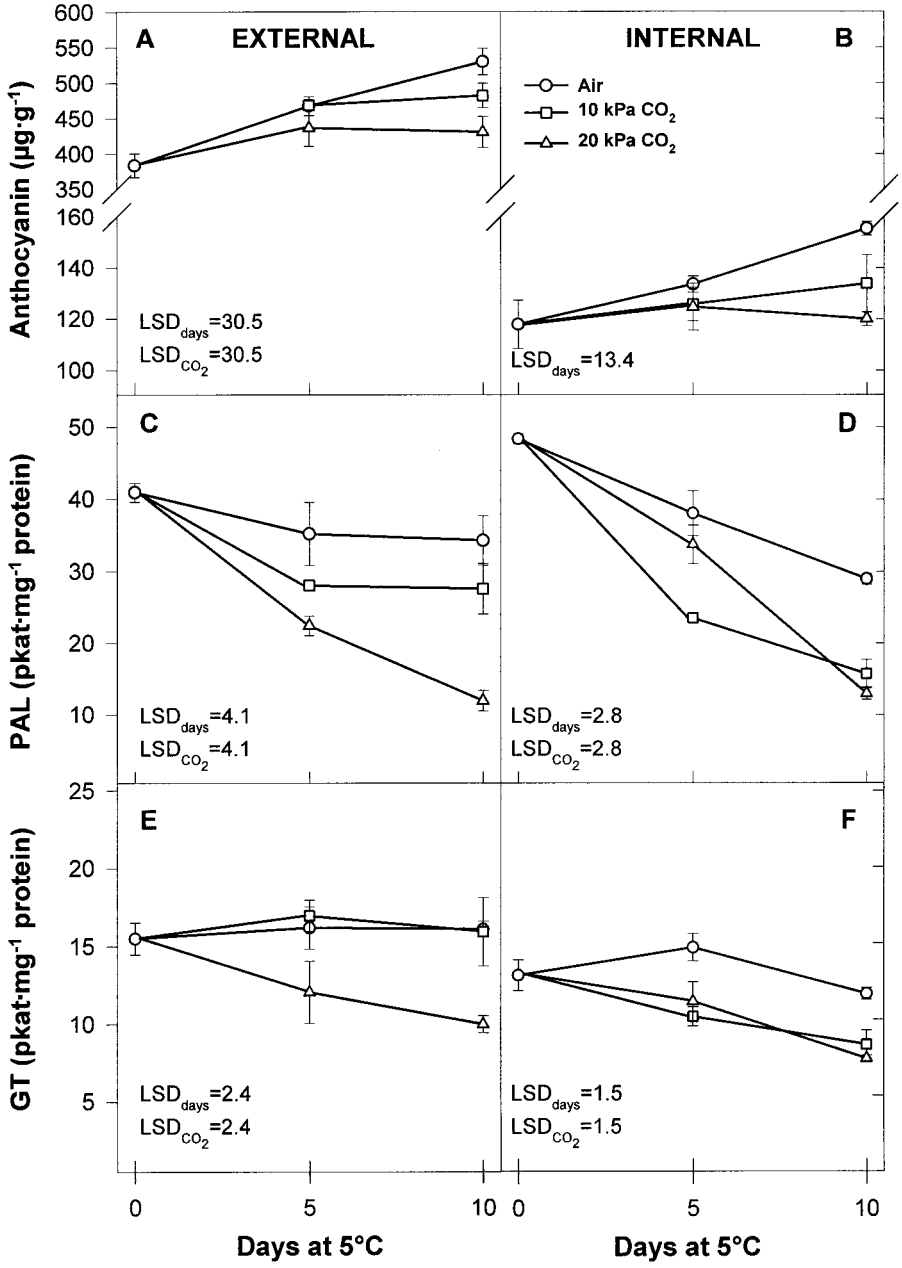

Fig. 2. Total anthocyanin concentration (µg·g^{-1}) (**A, B**) and activities of PAL (**C, D**) and GT (**E, F**) (ρkat·mg^{-1} protein) from the external and internal tissue of 'Selva' strawberries stored at 5 °C for 5 or 10 d in air or CO_2-enriched atmospheres.

nal and internal tissues. Cyanidin 3-glucoside was present in external tissues (8%) but completely lacking in the internal tissues. These differential concentrations help explain the large differences in hue angle between the skin and the flesh of the strawberry, since Pg derivatives are scarlet red while Cy derivatives tend to be crimson red. Changes in the relative percentage of each as a result of storage treatment were small, except in external tissues of fruit stored for 10 d in air + 20 kPa CO_2, where the concentration of Cy 3-G was lower and the concentrations of Pg derivatives were higher.

Enzyme activities (PAL and GT). Activity of PAL in the external tissues decreased during storage for all treatments ($P \leq 0.01$) and this decrease was significantly ($P \leq 0.001$) affected by the storage atmosphere (Fig. 2C). After 10 d, the activity of PAL from fruit stored in air + 20 kPa CO_2 was 11.9 ρkat·mg^{-1} protein while activity in the air-stored control was 34.3 ρkat·mg^{-1} protein. The correlation between anthocyanin concentration and PAL activity was not significant, but when anthocyanin concentration was low, PAL activity was also low (Fig. 2). A similar relationship was seen between GT activity and anthocyanin concentration. Activity of GT remained fairly constant in air-stored fruit but decreased in fruit stored in air + 20 kPa CO_2, whereas GT activity in fruit stored in air + 10 kPa CO_2 was similar to that in the air-stored fruit (Fig. 2E).

The activities of PAL and GT in the internal tissues were affected by both storage time and atmosphere ($P \leq 0.01$) (Fig. 2). The activities of both enzymes were always higher in air-stored fruit than in fruit stored in elevated CO_2 atmospheres. Activity of PAL in fruit stored in air + 20 kPa CO_2 for 10 d was 44.9% of that of the air-stored control, and GT activity was significantly ($P \leq 0.05$) lower after 10 d of storage in the CO_2-stored fruit (7.4 ρkat·mg^{-1} protein, in air + 20 kPa CO_2 vs. 11.7 ρkat·mg^{-1} protein in air) (Fig. 2F).

In 'Selva' strawberry fruit both PAL and GT activities were affected by CO_2-enriched atmospheres. This relationship differed from pomegranates, where the anthocyanin concentration was correlated to PAL activity, but GT activity was unaffected by storage atmosphere (Holcroft et al., 1998). Increases in the activities of PAL and GT have been positively correlated with anthocyanin production in several fruits, including strawberry (Cheng and Breen, 1991; Given et al., 1988). Given et al. (1988) found that activity of GT was about one-third of that of PAL, and we noted a similar difference. However, the activities of both enzymes were lower in our experiment, probably because collection of data was restricted to a 10-d postharvest period, whereas Given et al. (1988) measured activity during fruit development.

Phenolics. The total soluble phenolic concentration, measured as ρ-coumaric acid equivalents by Folin-Ciocalteau assay, or as the sum of the individually identified phenolic compounds, followed the same trends in both external and internal tissues (Table 1). The content of phenolic compounds increased with time ($P \leq 0.05$) in storage but was unaffected by storage atmosphere. It was significantly higher in external tissues than in internal tissues, with ellagic acid and flavonols (quercetin and kaempferol derivatives) present only in the external tissues, while catechin and ρ-coumaroyl glucose occurred throughout the fruit (Table 1). Generally, the concentrations of individual phenolics increased during storage, particularly in air. An increase in concentration after 5 d of storage in air + 20 kPa CO_2 was observed, although by 10 d the concentrations were usually lower than in the air control, indicating that degradation of phenolic compounds may increase after prolonged storage in elevated CO_2 atmospheres.

Differences in concentrations of phenolic compounds between the two methods may reflect the fact that HPLC specifically measures simple phenolics, whereas the Folin-

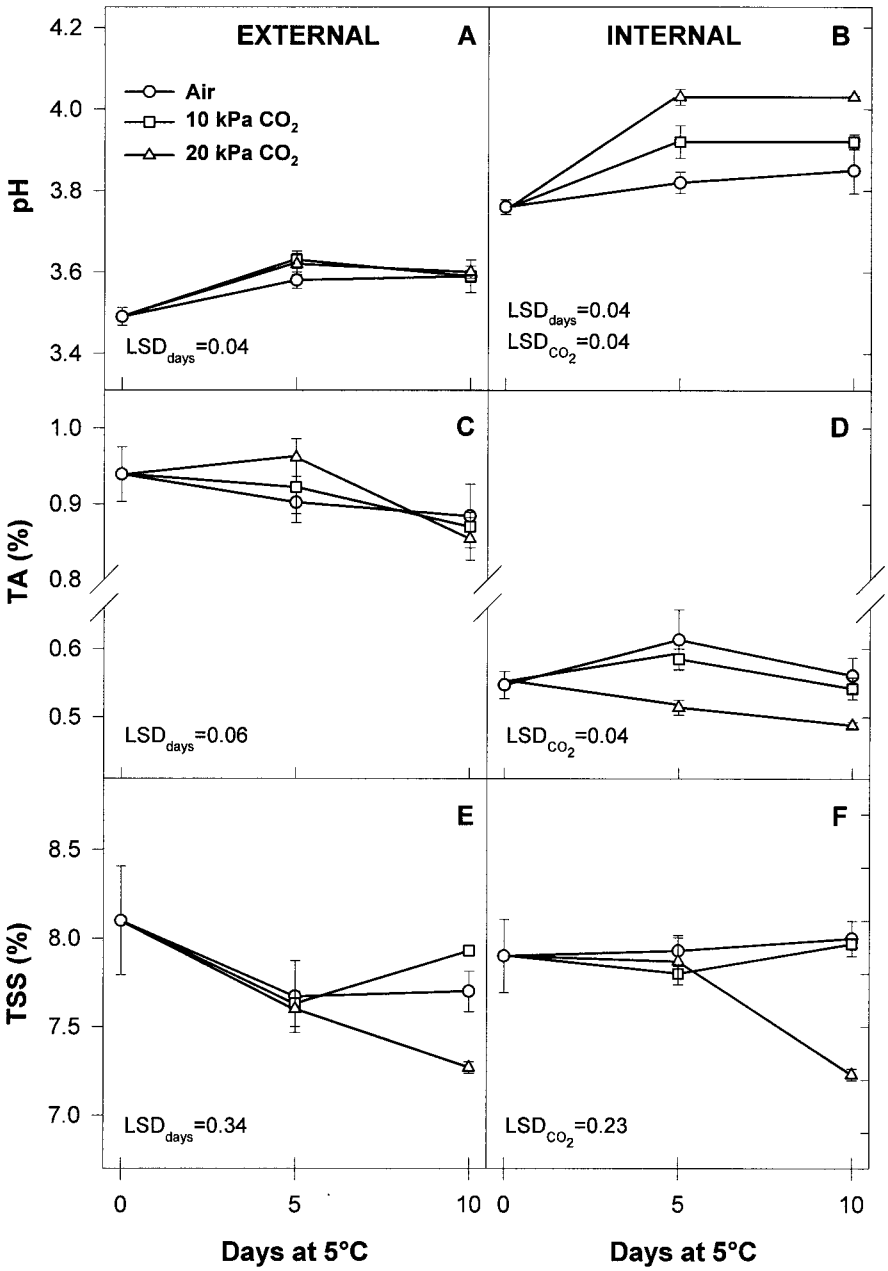

Fig. 3. pH (**A, B**), titratable acidity (TA) (**C, D**) and total soluble solids content (TSS) (**E, F**) of external and internal tissue of 'Selva' strawberries stored at 5 °C for 5 or 10 d in air or CO_2-enriched atmospheres.

Ciocalteau assay is a nonspecific method that measures simple phenolics, polyphenols, flavonoids, tannins, and some readily oxidized substances such as ascorbic acid (Singleton and Rossi, 1965).

Total protein. Extractable protein was lower in internal than in external tissue (2.7 vs. 3.2 $\mu g \cdot g^{-1}$, respectively) and increased by 0.4 $\mu g \cdot g^{-1}$ in both tissues ($P \leq 0.05$) after 10 d at 5 °C. However, this increase was not affected by storage atmosphere. These data indicate that storage in CO_2 atmospheres has no effect on net protein synthesis in strawberry, in contrast with tomato fruit, where Rothan et al. (1997) reported a 33% reduction in extractable protein in fruit stored for 2 d under 20% CO_2. Thus, the differences in enzyme activity found here could be ascribed to a more direct effect of CO_2 storage on the enzyme rather than an overall reduction in protein synthesis.

pH, TA, and TSS. Initially, the pH of the internal tissue was slightly higher than that of external tissue (3.76 vs. 3.49, respectively) (Fig. 3 A and B). External pH increased by only 0.1 unit during storage and the differences between the storage treatments were negligible after 10 d (Fig. 3A). During 10 d of storage the pH of the internal tissues increased by 0.27 pH unit in air + 20 kPa CO_2, vs. only 0.09 unit in air (Fig. 3B). The TA was considerably lower in internal (Fig. 3C) than external tissues (0.55% vs. 0.94%, respectively). In both tissues, TA decreased with time in storage. TA of the internal tissues of fruit stored for 10 d in air + 20 kPa CO_2 was lower than that of fruit stored in air (0.49% vs. 0.56%, respectively) (Fig. 3D).

Anthocyanin analysis by HPLC was conducted at a low pH to enhance the stability of the pigments, and this favors conversion to the red flavylium form. Consequently, the correlation between anthocyanin concentration and visual color may be poor. This overestimation of the red form of anthocyanin was seen in pomegranates (Holcroft et al., 1998), where anthocyanin values measured by HPLC analysis correlated poorly with those calculated from absorbance (510 nm) measured in a buffered dilution (pH 3.5).

The color reversal data show no recovery of internal fruit color in strawberry after 3 d in air, indicating that any normalization of pH caused by return to air did not affect color expression. Either there was no major effect of pH on color, or degradation of the chalcone had already occurred.

TSS did not differ significantly between internal and external tissues. Storage atmospheres did not affect TSS until after 10 d of exposure, when TSS was lower in both external and internal tissues of fruit stored in air + 20 kPa CO_2 (Fig. 3 E and F). Lewis et al. (1995) found that the absorbance of anthocyanins increased when glucose, sucrose or maltose (all 50% w/v) were added, although the absorbance maxima remained unchanged. This hyperchromic shift may be caused by a reduction in water activity by high sugar concentrations, causing the equilibrium to favor the flavylium ion. This effect was greater at pH 4 than at pH 2. The lower TSS values in the air + 20 kPa CO_2 treatment, in combination with higher pH, may also have affected anthocyanin stability.

Despite the benefits of CO_2-enriched atmospheres in controlling postharvest decay of strawberry fruit, these atmospheres adversely affected fruit color, anthocyanin concentration, and the activities of at least two enzymes in the anthocyanin biosynthetic pathway. The effects were more obvious in the internal tissues.

Table 1. Mean concentrations of phenolic compounds ($\mu g \cdot g^{-1}$) in external and internal tissues of 'Selva' strawberries stored at 5 °C for 5 or 10 d in air or air enriched with CO_2.

Days in storage	CO_2 (kPa)	Catechin	ρ-Coumaroyl glucose	Ellagic acid	Quercetin derivatives	Kaempferol derivatives	Total (sum)	Total (ρ-coumaric acid equiv.)
External tissue								
0	---	118	8	24	47	24	221	1705
5	0	160	13	22	54	30	279	1924
	10	171	10	22	55	29	287	1986
	20	190	11	20	52	27	300	2011
	Mean	174	11	21	54	29	289	1974
10	0	190	16	23	67	31	327	2040
	10	214	11	21	60	30	336	1957
	20	184	10	20	51	29	294	1869
	Mean	196	12	21	59	30	318	1955
LSD_{days} (5%)		21.5	1.8	NS	9.9	2.3	26.3	68
Internal tissue								
0	---	122	16	---[z]	---	---	138	966
5	0	154	21	---	---	---	175	1009
	10	151	18	---	---	---	169	1054
	20	167	19	---	---	---	186	1222
	Mean	157	19	---	---	---	176	1062
10	0	167	23	---	---	---	190	1121
	10	163	17	---	---	---	180	1093
	20	124	17	---	---	---	141	984
	Mean	151	19	---	---	---	170	1066
LSD_{days} (5%)		16.1	1.7				15.4	NS

[z]Not detected.

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