Behavior of Flavonols and Carotenoids of Minimally Processed Kale Leaves during Storage in Passive Modified Atmosphere Packaging

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Abstract: Minimally processed kale leaves were packed in passive modified atmosphere and stored at 3 conditions: 1 °C in the dark and 11 °C with or without light exposure. The products were evaluated during storage in terms of headspace gas composition, sensory attributes, flavonol, and carotenoid contents. The sensory quality decreased slightly during 17 d at 1 °C in the dark. At 11 °C, the vegetable shelf life was predicted to be 6 d in the dark and 3 d with light. Quercetin and kaempferol were stable during storage for 15 d at 1 °C in the absence of light. At 11 °C in the dark, quercetin was stable during 10 d, increasing slightly on the 8th day. Kaempferol decreased up to the 5th day but increased on the 8th day, decreasing again on the 10th day. After 5 d at 11 °C under light, the flavonol levels were significantly higher than those of the initial values. Neoxanthin and violaxanthin did not change significantly after 15 d at 1 °C in the dark. Lutein and β-carotene, however, decreased 7.1% and 11.3%, respectively. At 11 °C in the dark, neoxanthin, violaxanthin, lutein, and β-carotene decreased 16.1%, 13.2%, 24.1%, and 23.7% after 10 d, respectively. At 11 °C under light, neoxanthin and lutein had a slight increase while violaxanthin and β-carotene decreased 23.1% and 16.5% after 5 d.

Keywords: carotenoids, flavonols, kale leaves, minimal processing, modified atmosphere packaging

Practical Application: Passive modified atmosphere packaging together with refrigeration can extend the shelf life of minimally processed kale, retaining the health-promoting compounds, flavonols and carotenoids. Quercetin, kaempferol, neoxanthin, and violaxanthin are stable and lutein and β-carotene slightly reduced.

Introduction

Consumption of fruits and vegetables has been widely associated with lower incidence of degenerative diseases (Steinmetz and Potter 1996; Ness and Powles 1997; Liu and others 2001; Hung and others 2004). This protection against diseases has been attributed to bioactive compounds found in these plant foods, such as flavonoids and carotenoids (Rice-Evans and Miller 1995; Patil and others 2009). Flavonoids have been linked with lower risk of coronary heart diseases and cancer (Hertog and others 1997; Knekt and others 1997; Neuhouser 2004). Aside from their action against cancer and cardiovascular diseases, carotenoids also reduce the risk of macular degeneration and cataract (Moeller and others 2000; Renzi and Johnson 2008; Nishino and others 2009) and some carotenoids have provitamin A activity.

Fresh-cut or minimal processing is the current trend for marketing fruits and vegetables, stimulated by consumer's demand for convenience and for fresh-like qualities. These benefits, however, are offset by the rapid deterioration and short shelf life of these products and the potential health hazards associated with spoilage (Artes and others 2007). Cutting, peeling, or shredding destroys the natural protection of the epidermis and the compartmentation that separates enzymes from substrates, resulting in physical damage, microbial development, and enzymatic reactions that can render the processed product more perishable than the original intact produce. Respiratory activity and ethylene emission are generally increased (Varoquaux and Wiley 1994; Martínez and others 2005). To maintain freshness, extend shelf life, and ensure safety, low temperature and modified atmosphere packaging (MAP) are employed (Zagory 1998). Lowering the O_2 level and increasing the CO_2 level suppresses respiration, ethylene production, cut-surface browning, senescence, and growth of microorganisms (Wang 2006). Water loss is reduced because of high relative humidity within MAP.

Because thermal processing is not involved, minimal processing and MAP are expected to retain the nutrients and other bioactive compounds of vegetables and fruits. However, studies to demonstrate the effects of minimal processing and MAP on individual bioactive compounds, particularly in leaves, are still limited. With the current emphasis on the health-promoting effects of foods, investigations on this aspect are urgently needed.

The present study was carried out to evaluate the stability of carotenoids and flavonols in minimally processed kale during dark and lighted storage under passive modified atmosphere packaging. To the best of our knowledge, this is the first time that individual flavonols and carotenoids had been simultaneously monitored during storage of minimally processed leaves. Kale, a widely consumed leafy vegetable in Brazil, is a rich source of

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flavonols (quercetin and kaempferol) (Huber and others 2009) and carotenoids (lutein, β-carotene, violaxanthin, neoxanthin) (Azevedo-Meleiro and Rodriguez-Amaya 2005a).

Materials and Methods

Minimal processing

The kale leaves were processed in a small-scale industry located in São Roque, São Paulo, engaged in minimal processing of vegetables. The prewashed leaves were selected, trimmed, and washed with chlorinated water (100 ppm) and water at approximately 6 °C. These leaves were cut into 2 mm strips, sanitized by immersion in a solution of hypochlorite (200 ppm), washed again with water, and centrifuged at low speed. In Brazil, kale leaves are commonly cut into thin strips for cooking, thus minimally processed kale is cut in the same manner. These operations were carried out in a refrigerated room maintained at a temperature of about 15 °C. Portions of approximately 150 g were packed in expanded polystyrene trays (205 × 140 × 3.5 mm), wrapped with stretched polyvinyl chloride film (mean thickness of 11 μm after stretching); oxygen transmission rate was 12889 mL (STP)/(m^2.d) at 23 °C under dry condition. The samples were immediately transported, in isothermic boxes cooled with ice, to our laboratories for storage and sensory and chemical analyses.

Storage

The samples were stored at 3 different conditions: 1 °C in the dark and 11 °C without or with light (576 lux) exposure, the last 2 conditions being the common practice in Brazil. The samples exposed to light were in special shelves with 2 lamps (brand Osram, model 30W/765, daylight) of the same length as the shelves. The intensity of light over the product was measured with a luximeter, model 407026 (Extech Instruments Corp., Waltham, Mass., U.S.A.) with a resolution of 1 lux. During storage, headspace gas composition, sensory attributes (overall appearance, overall quality, discoloration, wilting, senescence, and undesirable odor), and carotenoid and flavonol concentrations were determined.

The flavonoid and carotenoid analyses were carried out while the sensory analyses indicated that the product was still acceptable. Thus, the leaves stored at 1 °C in the dark were analyzed at 1, 3, 5, 8, 10, 12, and 15 d of storage; those stored at 11 °C in the dark at 1, 3, 5, 8, and 10 d and under light at 1, 3, and 5 d of storage.

For each condition on each day of analysis, 3 packages were homogenized and subsamples (250 g) were then taken and weighed for flavonoid and carotenoid determinations. All analyses were carried out in triplicate.

Evaluation of the sensory quality

Alteration of the sensory quality was evaluated, using a structured scale of 5 points, by a panel of 10 untrained women, used to buying and cooking kale leaves. The scores for overall appearance were: 1—very bad, 2—bad, 3—regular, 4—good, 5—excellent. For discoloration, wilting, senescence, and undesirable odor the scores were: 1—absent, 2—slight, 3—moderate, 4—intense, 5—very intense. Each panel member was presented a package of the product for each treatment. This evaluation was done to estimate the shelf lives so as to ensure that the carotenoid and flavonoid analyses were carried out throughout the shelf lives. A score of 3 was considered the limit of acceptability of the product for each of the quality attribute evaluated.

Determination of gas composition

The O_2 and CO_2 levels in the package's headspace were determined using a Shimadzu gas chromatograph model 14A (Shimadzu Corp., Nakagyo-ku, Kyoto, Japan), equipped with a thermal conductivity detector operated at 140 °C, Porapak Q and molecular sieve 5A columns (Supelco Inc., Bellefonte, Pa., U.S.A.) at 82 °C, and an injector set at 84 °C. From each package, an aliquot of 0.5 mL of the headspace gas was hermetically withdrawn through a silicone septum adhered to the package's surface. The results were expressed as percentages in volume of gas.

Flavonoid analysis

A known amount of water (1 : 1, water : sample) and ascorbic acid (enough to give a final concentration of 0.04%) was added to the weighed sample and homogenization was undertaken for 3 min at 25000 rpm in a Polytron MR2100 homogenizer (Kinematica AG, Littau, LU, Switzerland). Using 7.5 g of the homogenized sample, the flavonols were quantified as aglycones according to a method optimized and validated by Huber and others (2007). Simultaneous extraction/hydrolysis was done with 50% aqueous methanol with 1 M HCl at 90 °C for 6 h. The optimum hydrolysis condition was determined by a Central Composite Rotational Design (CCRD) and Response Surface Analysis. The extract was cooled and filtered through a glass-sintered funnel, the volume was completed to 50 mL with methanol, and the solution was sonicated for 5 min. An aliquot of about 2 mL was filtered through a 0.45-μm PTFE syringe filter (Millipore, Carrigtwohill Co., Cork, Ireland); a 20-μL aliquot was injected into the liquid chromatograph.

A Waters liquid chromatograph model 2690 (Waters Corp., Milford, Mass., U.S.A.) was used, equipped with a Rheodyne injector (model 7725i), a photodiode array detector (Waters 996) set at 370 nm, and a Nova-Pak C18 column (4 μm, 3.9 × 150 mm), controlled by Software Millenium 3.20. The mobile phase consisted of methanol : water (both acidified with 0.03% formic acid) in a multilinear gradient, starting with 20 : 80, changing to 45 : 55 in 5 min, 48 : 52 in 17 min, returning to 20 : 80 in 20 min. The flow rate was 1 mL/min.

The identification of the flavonols was based on the retention times, co-chromatography with standards, and the UV spectra obtained with the photodiode array detector. Quantification was done by external standardization. The quercetin and kaempferol standards were obtained from Sigma Chemicals Co. (St. Louis, Mo., U.S.A.).

Carotenoid analysis

Using 3 g of the homogenized sample, the carotenoids were determined according to a method developed and evaluated for leafy vegetables by Kimura and Rodriguez-Amaya (2002) and validated using a lyophilized vegetable mix certified reference material by Kimura and others (2007).

The method consisted of extraction with cold acetone in the Polytron MR2100 homogenizer, for 1 min at 11000 rpm and filtration through a glass-sintered funnel. Extraction and filtration were repeated until the residue turned colorless. The carotenoids were transferred to about 50 mL petroleum ether : ethyl ether (2 : 1) by partition, in a separatory funnel with the addition of water. The ether solution was washed free of acetone, dried with anhydrous sodium sulfate, concentrated in a rotary evaporator, and brought to dryness under nitrogen. Prior to injection, the carotenoids were dissolved in 2 mL high-performance liquid chromatography (HPLC) grade acetone and filtered through the

0.22 μm PTFE syringe filter; a 10-μL aliquot was injected into the liquid chromatograph. All the necessary precautions were taken to avoid alterations or losses of the carotenoids and other errors during analysis.

Another Waters separation module, model 2690, was used, equipped with quaternary pump, autosampler injector, degasser, and a photodiode array detector (model 996), controlled by a Millenium 3.20. Detection was at the wavelengths of maximum absorption (max plot).

The column was monomeric C_{18} Waters Spherisorb ODS2 (3 μm, 4.6 × 150 mm). The mobile phase consisted of acetonitrile (containing 0.05% of triethylamine), methanol, and ethyl acetate, used at a flow rate of 0.7 mL/min. A concave gradient (curve 10) was applied from 95 : 5 : 0 to 60 : 20 : 20 in 20 min, maintaining this proportion until the end of the run. Reequilibration took 15 min.

Identification of the carotenoids was done according to Rodriguez-Amaya (1999), with the combined use of retention time, co-chromatography with standards, and the visible absorption spectra. Leafy vegetables are known to have the same qualitative composition, especially of the principal carotenoids. The identity of these carotenoids in kale, endive, and New Zealand spinach was confirmed by HPLC-MS by Azevedo-Meleiro and Rodriguez-Amaya (2005a, 2005b).

Quantification was by external standardization. Standards were isolated from a leafy vegetable (roquette) by open column chromatography on MgO : Hyflosupercel (1 : 1, activated for 4 h at 110 °C) packed to a height of 20 cm in 2.5 cm i.d. × 30 cm glass column. This column was developed with increasing amounts of ethyl ether and acetone in petroleum ether; the purity of the carotenoid isolates was monitored by HPLC. The mean purity of the standards was 95% for neoxanthin, 96% for violaxanthin, 97% for lutein, and 95% for β-carotene. The concentrations of the standard solutions were corrected accordingly.

In both flavonoid and carotenoid analyses, the standard curves were constructed by the injection in triplicate of standard solutions at 5 different concentrations. The curves passed through the origin and were linear at the concentration range expected of the samples, the coefficients of correlation obtained being higher than 0.99.

Statistical analysis

To verify the existence of statistically significant differences, the results of the flavonoid and carotenoid analyses were submitted to analysis of variance (ANOVA; $P < 0.05$), the means being compared by the Tukey test, utilizing the GraphPad Prism 2.01 program.

Results and Discussion

Gas composition in the package

Figure 1 shows the gas levels (O_2 and CO_2) in the packages during storage. The variation in gas levels seen in this figure, especially at low temperature, is expected of fresh plant produce, intrinsic to plant physiology. In any case, a passive modification of the atmosphere inside the package can be noted, as a function of respiration of the product and permeability of the packaging. At 1 °C in the dark, an equilibrium atmosphere was established in the package from the second day (9% to 15% O_2 and 4% to 10% CO_2). The effect of storage temperature on gas composition was evident. At 11 °C in the dark, O_2 level fell to 0.2% and CO_2 reached 13% after 10 d. This indicated that the temperature had a greater effect on the produce respiration rate than on the package gas transmission rate, accelerating the modification of the atmosphere inside the package.

The effect of light can also be verified in kale stored at 11 °C. After 4 d of storage in the presence of light, O_2 was reduced to 0.3% and CO_2 increased to 21%. In the dark, modification of the atmosphere was less intense, the levels of O_2 and CO_2 staying at around 7%, in the same period. Exposure of the leaves to light (576 lux) apparently increased the metabolism of the leaves, accelerating the modification of the passive atmosphere in the package.

Sensory quality

Figure 2 shows the results of the sensory evaluation. Through the overall appearance, development of undesirable odor and discoloration, the shelf life of the samples stored at 1 °C in the dark was estimated to be 17 d. During this period, wilting and senescence continued to be within the acceptable range. For the leaves stored at 11 °C in the dark, the shelf life was 6 d, based on the overall appearance and discoloration. At the same temperature in the presence of light, the limit of acceptability for overall appearance and undesirable odor was reached on the 3rd day.

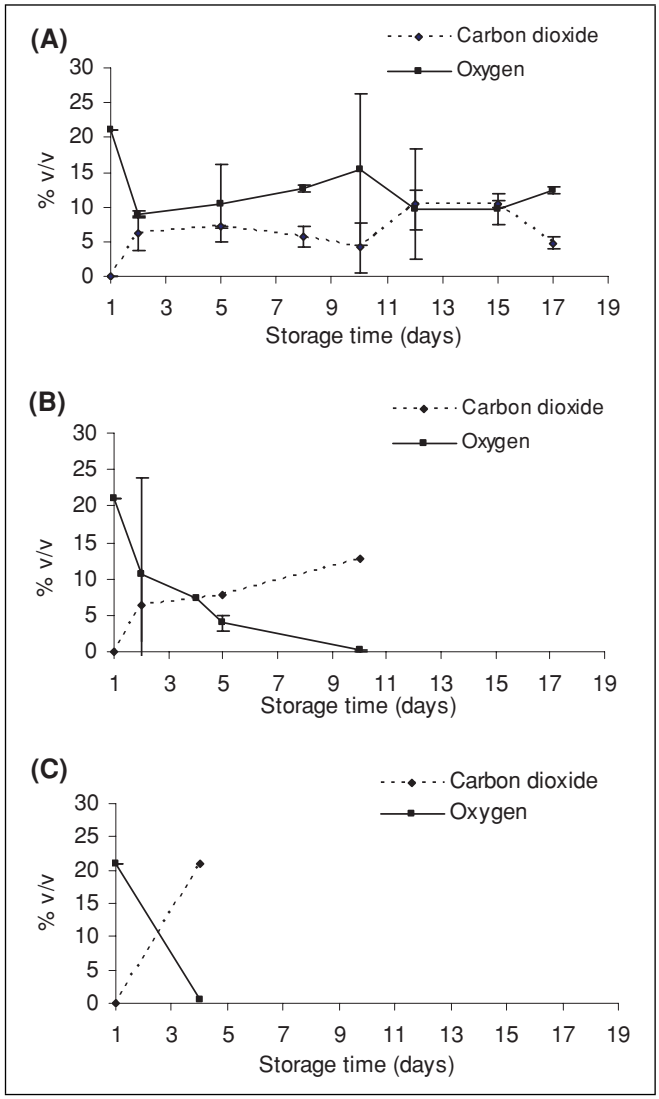

Figure 1–Evolution of the oxygen and carbon dioxide levels in the atmosphere of the packages of minimally processed kale during storage at (A) 1 °C in the dark, (B) 11 °C in the dark, (C) 11 °C under light.

Flavonol levels during storage

Typical HPLC chromatograms of the flavonols and carotenoids of kale are shown in Figure 3. Figure 4 shows the flavonol levels during storage of the minimally processed kale.

The flavonols, quercetin and kaempferol, in the kale samples were stable during 15 d of storage at 1 °C in the absence of light, with kaempferol increasing slightly on the 15th day. At 11 °C in the dark, quercetin had a slight increase on the 8th day of storage, but went back to the initial level in 10 d. Kaempferol decreased up to the 5th day, increased on the 8th day and decreased again on the 10th day. The quercetin and kaempferol levels in the samples stored at 11 °C under light remained the same on the 1st and 2nd day, but were statistically higher than the initial concentrations on the 5th day.

To ensure that the changes in the levels of flavonoids and carotenoids during storage were not due to changes in the moisture content of the leaves, the moisture content was determined in the initial samples and during storage. It was maintained at 90% ± 0.5%.

In fresh-cut Swiss chard, the total flavonoid content increased in both MAP (7% O_2 and 10% CO_2) and air-stored samples during 8 d at 6 °C, the increase being more significant in the former (Gil and others 1998). In contrast, vitamin C decreased, especially in the MAP-stored leaves, reaching levels 50% lower than the initial content. These same researchers found that the total flavonoid content in fresh-cut spinach remained stable during 7 d of storage at 10 °C in packages with air or modified atmosphere (Gil and others 1999). Vitamin C was better preserved in MAP-stored spinach.

Also investigating spinach, Bottino and others (2009) found that the total flavonoid content remained practically constant in the intact leaves and increased slightly in fresh-cut leaves during cold storage.

Ferreres and others (1997) observed that the quercetin glycoside content of minimally processed red pigmented lettuce packed in perfurated polyethylene bags, stored in a small room with humidified air at 5 °C, was stable in the white and green tissues during 14 d. In red tissues, where the flavonoid level was more elevated, there was an increase in 7 d, declining thereafter up to the 14-d storage period. The anthocyanin content decreased in both green and red tissues. In lamb's lettuce, free and total phenols increased in both control (intact) and fresh-cut leaves (Ferrante and others 2009). The total phenol content increased faster in cut leaves after 5 d, but was 23% higher in the control after 8 d of storage at 4 °C.

Carotenoid levels during storage

Figure 5 presents the carotenoid levels during storage. Neoxanthin and violaxanthin did not change significantly, but lutein and β-carotene decreased 7.1% and 11.3%, respectively, after 15 d at 1°C in the dark. At 11 °C in the dark, neoxanthin, violaxanthin, lutein, and β-carotene decreased 16.1%, 13.2%, 24.1%, and 23.7% after 10 d, respectively. At 11 °C under light, neoxanthin, and lutein had a slight increase, while violaxanthin and β-carotene decreased 23.1% and 16.5%, respectively, after 5 d.

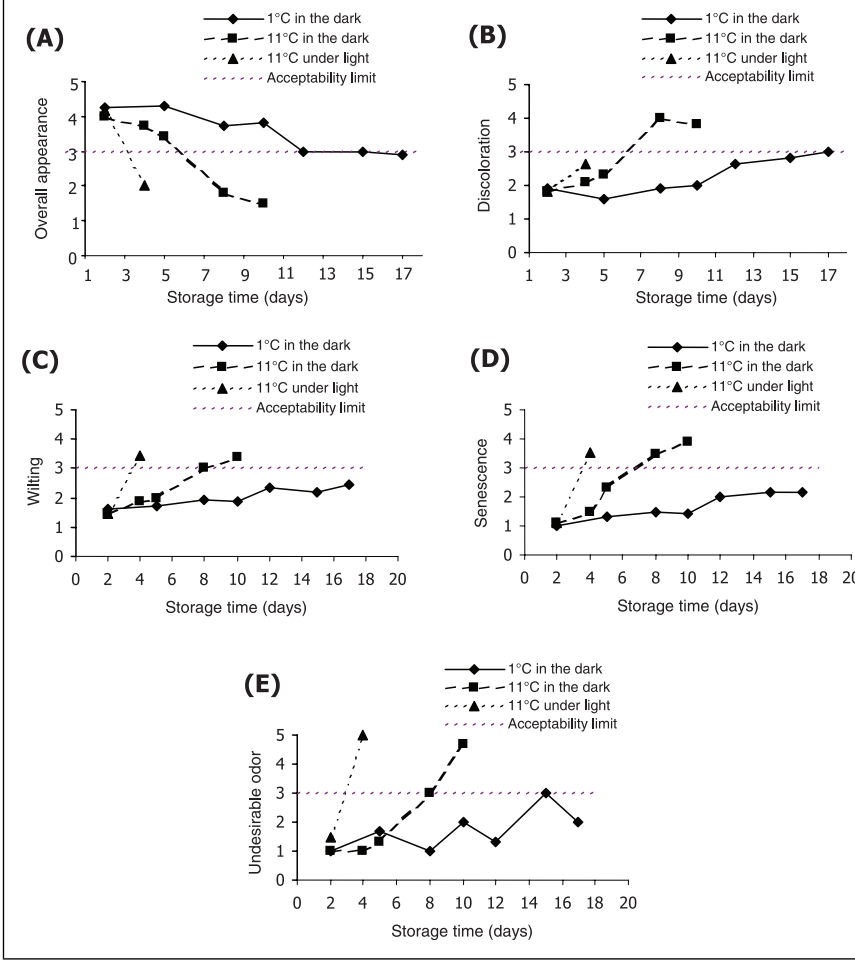

Figure 2–Sensory quality of minimally processed kale during storage under different lighting condition and temperature (A) overall appearance; (B) discoloration; (C) wilting; (D) senescence; (E) undesirable odor. The y-axes scores are described in the text.

The effect of light can be observed in the 23.1% reduction of violaxanthin in 5 d and the appearance of zeaxanthin (Figure 3), indicating that the xanthophyll cycle was functioning. This cycle, which is believed to have a role in photoprotection in plants, involves the de-epoxidation of violaxanthin to zeaxanthin under light and the epoxidation of zeaxanthin to violaxanthin under limiting light (Young and others 1997; Demmig-Adams 1990).

In minimally processed leafy vegetables (kale, endive. and New Zealand spinach) stored in polyethylene bags for 5 d at 7 to 9 °C, losses of 14% to 42%, 19% to 32%, 12% to 20%, and 8% to 31% were observed for β-carotene, lutein, violaxanthin, and neoxanthin, respectively, greater losses being observed in New Zealand spinach with the exception of neoxanthin, which degraded more in kale (Azevedo-Meleiro and Rodriguez-Amaya 2005a, 2005b).

Carnelossi and others (2002) investigated the effect of temperature (1, 5, and 10 °C) and type of packaging (different permeability to O_2 and CO_2 and PET trays) on minimally processed kale stored for 15 d. The total carotenoid content remained stable during the storage period at the 3 temperatures studied. However, it was less stable when the vegetable was packed in PET trays and there was a slight increase when the high permeability package was used at 1 °C storage. Vitamin C decreased with storage, especially in more permeable packaging.

Ferrante and others (2008) studied quality changes during storage of fresh-cut or intact Swiss chard leafy vegetables under dark or lighted storage until 12 d at 5 °C. While anthocyanin content strongly decreased in cut leaves in the dark and under light, total carotenoids did not significantly decline. These same researchers

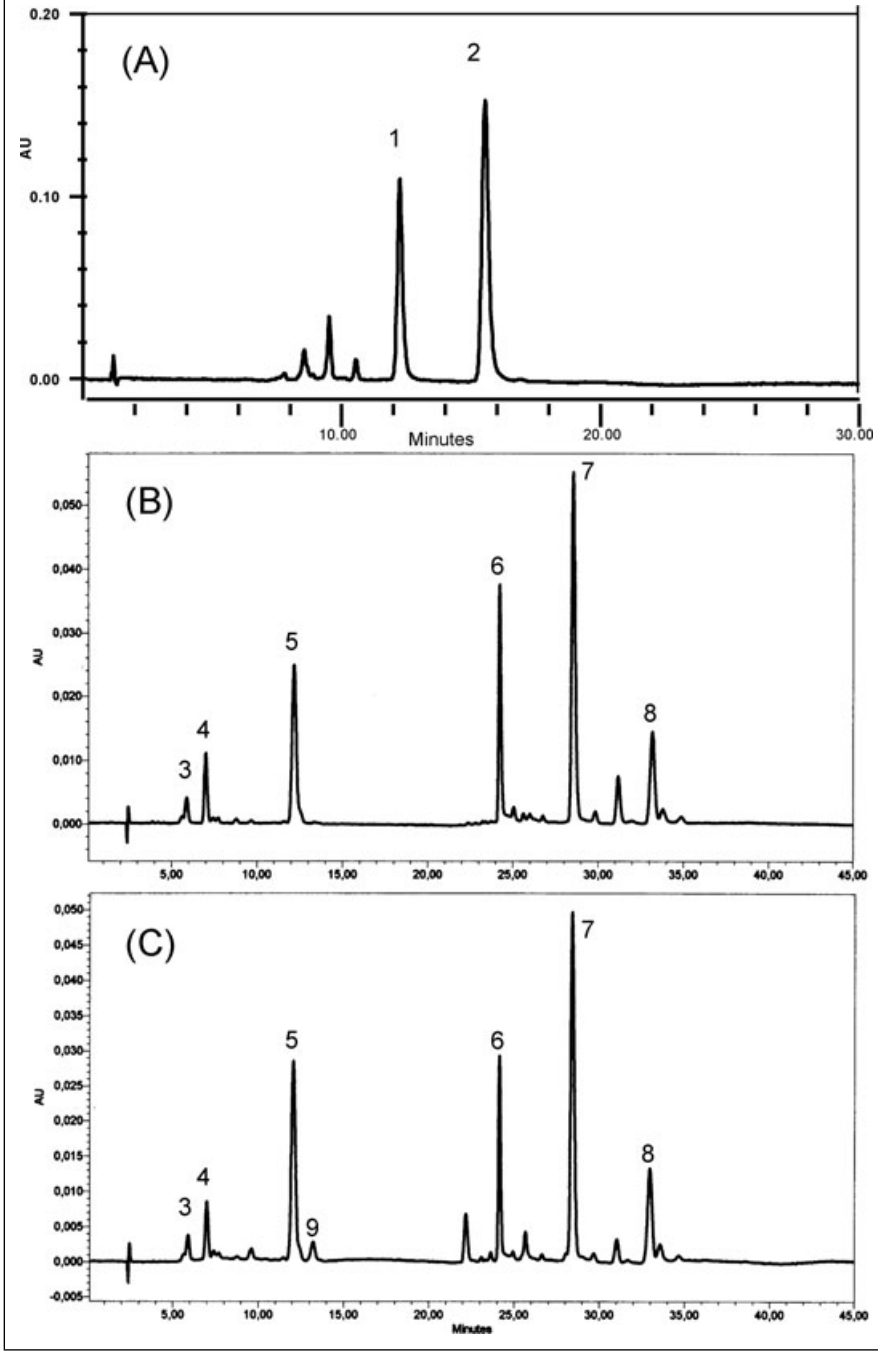

Figure 3–Typical HPLC chromatograms of (A) flavonols and (B; C) carotenoids of minimally processed kale. Peak identification: 1. quercetin; 2. kaempferol; 3. neoxanthin; 4. violaxanthin; 5. lutein; 6 and 7. chlorophylls; 8. β-carotene; 9. zeaxanthin. Chromatographic conditions are described in the text.

(Ferrante and other 2009) evaluating cut and intact lamb's lettuce leaves reported an increase in anthocyanins and a decrease in total carotenoids from 20 to 16 mg/g of after 8 d of storage at 4 °C in darkness in both treatments.

With minimally processed lettuce, Martin-Diana and others (2007) verified the effect of steamer jet-injection as an alternative to the usual chlorine sanitizing treatment. Significant reduction of ascorbic acid content and, to a lesser extent, of the total carotenoid concentration during storage for 10 d at 4 °C was observed. In the lettuce treated with chlorine, the carotenoid level was maintained at 13 μg/g whereas the lettuce exposed to vapor had only one-third of this concentration. In 2008, these same researchers (Rico and others 2008) optimized the short time blanching (steaming) by Response Surface Methodology. It was concluded that steamer treatment of 10 s could be considered the optimum time for maintaining the shelf life (mainly texture and browning) of fresh-cut lettuce for 7 to 10 d in optimum conditions. However, the use of the steamer even for very short time (5 s) significantly reduced the ascorbic acid and carotenoid contents of the samples.

Our results together with those of other researchers reveal a tendency of flavonoids to remain stable or increase at some points while carotenoids tend to maintain their levels or decrease during storage of minimally processed leaves. Fluctuations or seemingly inconsistent levels of these phytochemicals can be explained

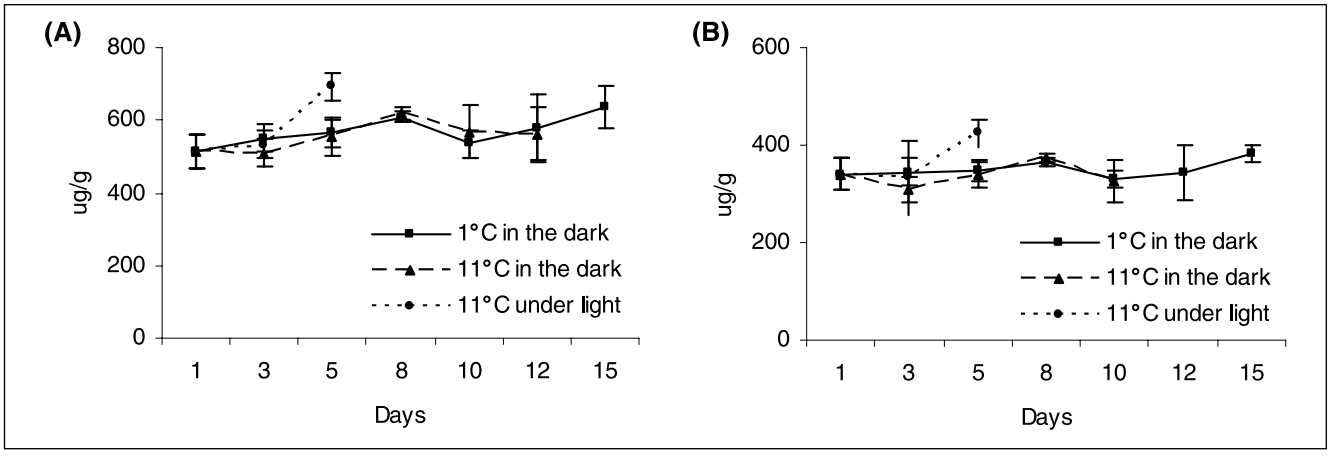

Figure 4–Concentrations of (A) quercetina e (B) kaempferol in minimally processed kale during storage at 1 °C in the dark and at 11 °C without and with light exposure.

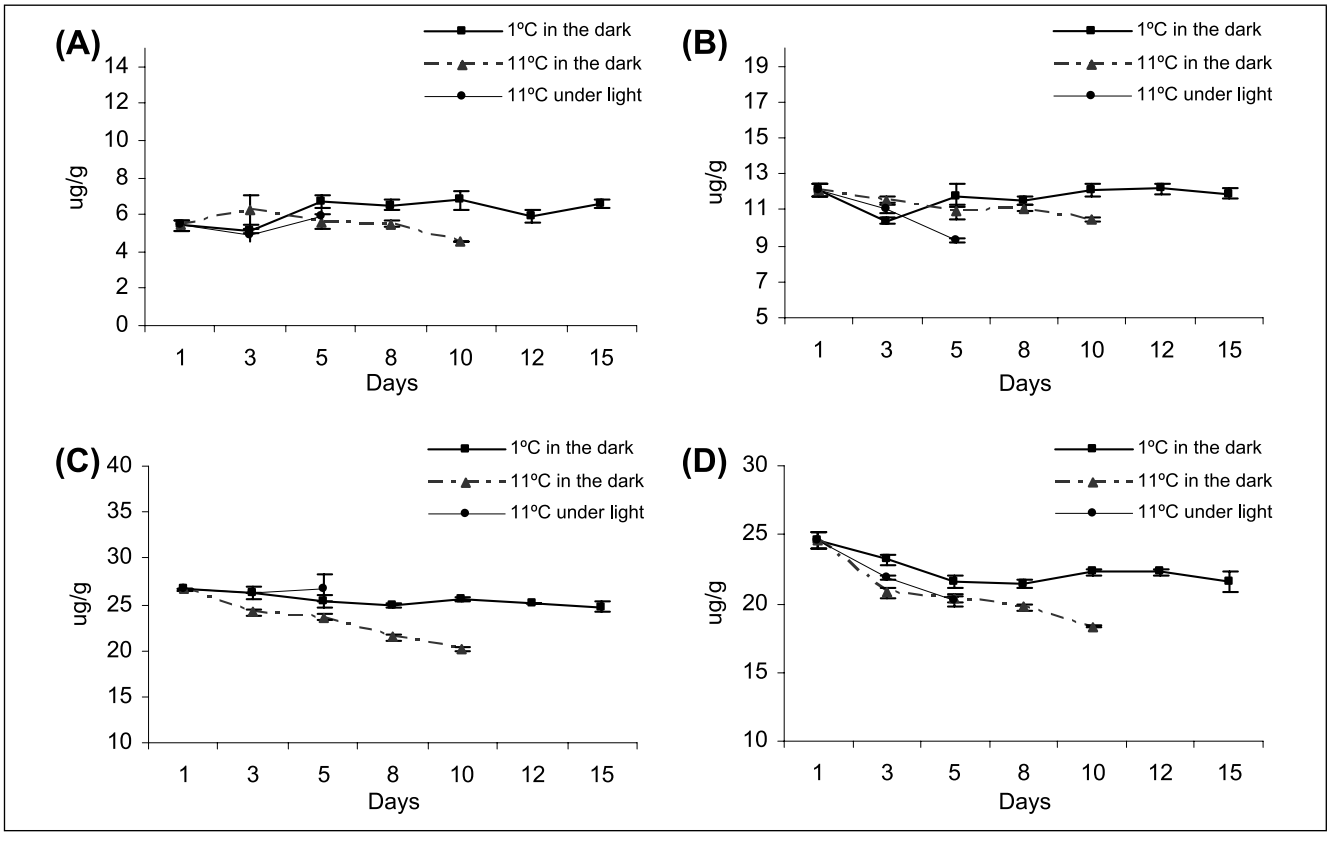

Figure 5–Concentrations of (A) neoxanthin, (B) violaxanthin, (C) lutein, and (D) β-carotene of minimally processed kale during storage at 1 °C in the dark and at 11 °C without and with light exposure.

by the possible occurrence of processes with opposing effects on their concentrations. Because thermal processing is not involved, the biosynthetic enzymes may remain active, increasing the phytochemical's level. On the other hand, cutting/shredding the vegetables may destroy compartmentation of oxidative enzymes, which can then promote degradation, especially of the carotenoids. Temperature elevation and exposition to light increase biosynthesis but also accelerate degradation. The concentrations of the phytochemicals at any one time would reflect which of the 2 processes is predominating.

Increases in flavonoid content can also be explained by another phenomenon. Wounding brought about by cutting, chopping, or shredding induces the synthesis of the enzymes of the phenylpropanoid pathway, subsequent synthesis, and accumulation of protective phenolic compounds and tissue browning (Bolin and Huxsoll 1991; Lopez-Galvez and others 1996; Tomás-Barberán and others 1997). Discoloration was not observed in the present study probably because one of the benefits of MAP is to prevent this cut-surface browning.

Conclusions

Passive modified atmosphere packaging together with refrigeration extended the shelf life of minimally processed kale. Quercetin and kaempferol were stable during storage, tending to increase at some points, especially under light exposure. Neoxanthin and violaxanthin were also stable at low temperature (1 °C) in the dark, but lutein and β-carotene were slightly reduced. At higher temperature (11 °C) in the dark, all 4 major carotenoids decreased with greater losses of lutein and β-carotene. Under light, violaxanthin loss was greater, followed by β-carotene, while neoxanthin and lutein increased slightly.

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