# Effect of Postharvest Storage and Processing on the Antioxidant Constituents (Flavonoids and Vitamin C) of Fresh-Cut Spinach

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The effect of modified atmosphere packaging (MAP) and cooking on the flavonoids and vitamin C content (ascorbic + dehydroascorbic acid; AA + DHAA) of fresh-cut spinach was evaluated. The total flavonoid content (approximately 1000 mg $kg^{-1}$ f.w.) remained quite constant during storage in both air and MAP atmospheres, while vitamin C (750 mg $kg^{-1}$f.w.) was better preserved in MAP-stored spinach. AA was transformed to DHAA during storage, and its concentration was higher in MAP-stored tissues. The free-radical scavenging activity of the isolated flavonoids was tested, and only those flavonoids with either a dihydroxyl grouping or acylated with ferulic acid showed significant activity. A decrease in the total antioxidant activity was observed during storage, particularly important in MAP-stored spinach. The higher content of DHAA and lower content of both AA and antioxidant flavonoids in the MAP-stored samples could explain this antioxidant activity decrease. Boiling extracted 50% of total flavonoids and 60% vitamin C in the cooking water. However, flavonoid glucuronides were extracted more in the cooking water than the other glycosides. The vitamin C content of the cooked tissue was higher in those samples stored in MAP.

**Keywords:** *Spinacia oleracea; spinach; flavonoids; vitamin C; antioxidants; modified atmosphere packaging; cooking; minimally processed; fresh-cut*

## INTRODUCTION

As part of our studies on the nutritional importance of fruit and vegetables, we investigate the flavonoids and vitamin C content of these products and their changes with postharvest treatments (modified atmospheres, minimal processing, domestic cooking, etc). There is considerable evidence for the role of antioxidant constituents of fruits and vegetables in the maintenance of health and disease prevention (Ames et al., 1993). Recent work is also beginning to highlight the relation of flavonoids and other dietary phenolic constituents to these protective effects. They act as antioxidants by virtue of the free-radical scavenging properties of their constituent hydroxyl groups. The extended conjugation across the flavonoid structure and an increasing number of the hydroxyl groups enhance the antioxidant properties, allowing them to act as reducing agents, hydrogen- or electron-donating agents, or single-oxygen scavengers (Kanner et al., 1994; Salah et al., 1995; Vinson and Hontz, 1995).

Spinach is one important dietary vegetable usually consumed after cooking in boiling water either fresh or frozen. In the past few years, ready to use fresh-cut spinach has become more popular. Available fresh-cut spinach in the market is already cut and bagged in polypropylene films. Modified atmospheres with controlled concentrations of $CO_2$ and $O_2$ have been used to maintain the quality of fresh-cut spinach and ascorbic acid (AA) retention (McGill et al., 1966; Brugheimer et al., 1967; Izumi et al., 1997).The occurrence of at least 10 flavonoid glycosides has been reported in spinach. These are glucuronides and acylated di- and triglycosides of methylated and methylendioxiderivatives of 6-oxygenated flavonols (Aritomi and Kawasaki, 1984; Aritomi et al., 1986; Ferreres et al., 1997). However, changes occurring in these compounds during postharvest storage and processing (cooking) have never been tested and quantified.

It has been suggested that fresh-cut spinach should be stored at 0 °C (Izumi et al., 1997); however, it is often held at higher temperatures, especially during commercial displaying. Izumi et al. (1997) in previous studies determined the beneficial effect of low $O_2$ atmospheres on spinach leaves stored at 10 °C for 9 days. We have conducted similar storage time−temperature experiments to study the effect of modified atmosphere packaging (MAP) on the antioxidant metabolites in fresh-cut spinach.

As the consumption of fresh-cut spinach increases, it is important to take into account that processing (minimal processing or cooking) may affect antioxidant phenolics and vitamin C content. To minimize the degradation of these antioxidant metabolites, it is essential to know the variation during storage and after postharvest processing. The purpose of the present work was the quantification of these flavonoids in fresh-cut spinach and the study of the changes produced in individual compounds during postharvest storage and processing. In addition, the antioxidant capacity of spinach flavonoids has been determined by the free-radical scavenging assay.

## MATERIALS AND METHODS

**Plant Material.** Fresh-cut spinach from a major vegetable processor (Vega Mayor S.A., Navarra, Spain) was evaluated. Packages of 400 g were delivered refrigerated to the laboratory after purchasing through a produce wholesaler less than 12 h from processing. In some samples, an air atmosphere was created in the bag by perforating the film with a needle. Samples were analyzed in triplicate (three bags per treatment) initially and after 3 and 7 days of storage at 10 °C. Experiments were repeated 3 times with similar results.

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**Package Atmosphere.** The gas composition in the packages was monitored daily through a septum using a 1 mL syringe. The $CO_2$ and $O_2$ concentrations were determined by injection of 0.5 mL of a gas sample on a Perkin-Elmer (Ct) autosystem gas chromatograph equipped with a thermal conductivity detector (TCD).

**Extraction Procedure.** A 20 g fresh weight (f.w.) sample was homogenized with 80 mL of MeOH/$H_2O$ (5:95) plus citric acid (0.5 g/L) with EDTA (0.5 g/L). The homogenate was filtered through cheesecloth, and the extract was used for the evaluation of flavonoids and free-radical scavenging activity. After passing the filtrate through a $C_{18}$ Sep-Pak cartridge (Waters, Milford, MA), vitamin C was evaluated.

**Cooking.** Twenty grams of fresh-cut spinach, from both air and MAP, were submitted to boiling with 80 mL of water for 10 min at 90 °C to simulate domestic processing. The cooking water was filtered through filter paper Whatman N° 1 and adjusted to the initial volume with distilled water.

The effect of domestic processing was also evaluated on the plant material remaining. The cooked spinach was homogenized with 80 mL of extraction solution, MeOH/$H_2O$ (5:95), plus citric acid (0.5 g/L) with EDTA (0.5 g/L), and prepared the same as the extraction protocol.

**Flavonoid Analyses.** The extract was filtered through a 0.45 $\mu$m pore filter, and 20 $\mu$L was injected in a HPLC for identification and quantification of flavonoid compounds. Separations were achieved on a LiCrochart column (RP-18, 12.5 × 0.4 cm, 5 $\mu$m particle size) (Merck, Darmstadt, Germany). Elution was performed using water−formic acid (19:1, v:v) (A) and HPLC-grade methanol (B) as the mobile phases, on a gradient starting with 10% B in A to reach 40% B at 30 min and 80% B at 40 min. The flow rate was 1 mL $min^{-1}$, detection was achieved with a diode-array detector, and chromatograms were recorded at 280 and 350 nm.

**Vitamin C Determination.** Ascorbic acid and dehydroascorbic acid (DHAA) contents were determined as described by Zapata and Dufour (1992). The HPLC analysis of total vitamin C (AA + DHAA) was achieved after derivatization of DHAA into the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride (OPDA). The samples were analyzed with a Merck-Hitachi (Tokyo, Japan) liquid chromatograph equipped with a L-4000 UV detector and a L-6000 pump. Separations of DFQ and AA were achieved on a Kromasil 100 C-18 column (25 × 0.4 cm; 5 $\mu$m particle size; Tecnokroma, Barcelona, Spain). The mobile phase was methanol−water (5:95, v/v) containing 5 mM cetrimide and 50 mM potassium dihydrogen phosphate at a pH 4.5. The flow rate was 0.9 mL $min^{-1}$. The detector wavelength was initially set at 348 nm, and after elution of DFQ, it was manually shifted to 261 nm for AA detection. Standard solutions, column conditioning, and derivatization procedures have been previously described (Gil et al., 1998a).

To study the effect of heat on the AA and DHAA stability, a model solution containing 5 mg of AA and 5 mg of DHAA was prepared. Three replicates of 20 mL of solution were dissolved following the extraction protocol. Other three replicates were boiled in water for 10 min at 90 °C to simulate the same domestic processing as that of the plant material. Both model solutions (boiled or not) were compared and quantified by HPLC.

**Free-Radical Scavenging Assay.** The effect of AA and DHAA standards, flavonoids, and extracts on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was estimated according to the procedure described by Brand-Williams et al. (1995).

**Statistical Analyses.** The results were submitted to the analysis of variance when appropriate and the mean values compared using Duncan's multiple range test at 5% level.

## RESULTS AND DISCUSSION

**Flavonoid and Vitamin C Content and Free-Radical Scavenging Activity of Fresh-Cut Spinach.** The HPLC analysis of fresh-cut spinach extracts revealed the presence of 10 flavonoids (**1**−**10**) (Figure 1) which had been previously isolated and identified (Aritomi and Kawasaki, 1984; Aritomi et al., 1986; Ferreres et al., 1997). In this case, compound **7** (spinacetin-3-*O*-$\beta$-D-(2″-feruloylglucopyranosyl) (1→6)-$\beta$-D-glucopyranoside) was present in very small amounts, which prevented its quantification. The addition of the individual flavonoids was quoted as total flavonoid content. Fresh-cut spinach contained 1000 mg of total flavonoids per kilogram of fresh weight (f.w.). Compared with other flavonoid-rich vegetables such as red lettuce (1400 mg $kg^{-1}$, Gil et al., 1998b), Swiss chard (2700 mg $kg^{-1}$, Gil et al., 1998a), and red-onion (900 mg $kg^{-1}$, Ferreres et al., 1996), spinach showed a notable flavonoid content.

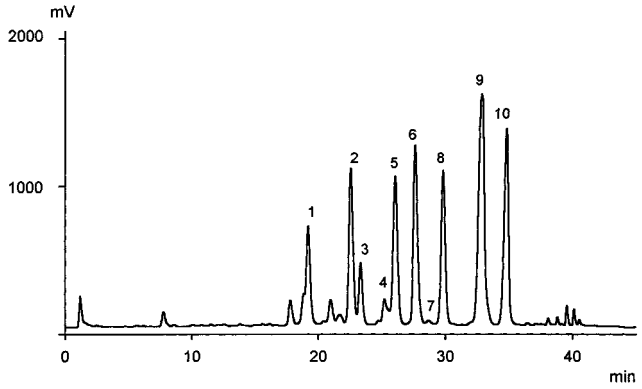


**Figure 1.** HPLC chromatogram of fresh-cut spinach flavonoids. See Table 1 for flavonoid identification.

The vitamin C content of fresh-cut spinach was 750 ± 50 mg $kg^{-1}$ f.w. Preliminary studies conducted with the same cultivar earlier in the season revealed higher amounts of vitamin C (1230 ± 50 mg $kg^{-1}$). Compared with the vitamin C content of other vegetables such as red lettuce (180 mg $kg^{-1}$), Swiss chard (440 mg $kg^{-1}$, Gil et al., 1998a), and onion (250 mg $kg^{-1}$, Erdman and Klein, 1982), its contribution to the diet is important.

The free-radical scavenging activity of the flavonoids isolated from spinach was compared with that of trolox, a synthetic analogue of vitamin E (Brand-Williams et al., 1995). The most active products were those derived of patuletin (3,5,7,3′,4′-penthahydroxy-6-methoxyflavone) which had a 3′,4′-dihydroxyl grouping (Table 1). Spinacetin (3,5,7,4′-tetrahydroxy-6,3′-dimethoxyflavone) derivatives showed almost no activity. The incorporation of a feruloyl residue, as in compounds **5** and **7**, increased the free-radical scavenging activity. The antioxidant activity of ferulic acid has been previously reported (Meyer et al., 1998). When the hydroxyl at the 4′-position was blocked, as in the case of compounds **8**, **9**, and **10**, no radical scavenging activity was observed, in accordance with previous reports (Williamson et al., 1996).

**Effect of Modified Atmosphere Packaging on Flavonoids and Vitamin C.** The packaging atmosphere was modified during storage to reach 12% $O_2$ + 7% $CO_2$ on day 3 and 6% $O_2$ + 14% $CO_2$ on day 7, as a result of the respiration rate of the tissue and the gas diffusion characteristics of the film at 10 °C.

After storage, total flavonoid content remained very stable both in air and MAP and no degradation during the storage period was observed (Table 3). These results were consistent with those previously reported on Swiss chard (Gil et al., 1998a). The stability of flavonoid compounds contrasts with that of hydroxycinnamic acids and anthocyanins (Gil et al., 1998b).

**Table 1. Free-Radical Scavenging Activity of the Individual Flavonoids in Fresh-Cut Spinach[a]**

| no. | aglycone | activity[b] |
|---|---|---|
| **1** | patuletin | 0.94 |
| **2** | spinacetin | 0.05 |
| **3** | patuletin | 0.62 |
| **4** | spinacetin | 0.00 |
| **5** | spinacetin | 0.28 |
| **6** | spinacetin | 0.03 |
| **7** | spinacetin | 0.20 |
| **8** | jaceidin | 0.00 |
| **9** | | 0.00 |
| **10** | | 0.00 |
| trolox | | 1.00 |

[a] (**1**) Patuletin 3-*O*-*β*-D-glucopyranosyl(1→6)-[*β*-D-apiofuranosyl(1→2)]-*β*-D-glucopyranoside; (**2**) spinacetin 3-*O*-*β*-D-glucopyranosyl(1→6)-[*β*-D-apiofuranosyl(1→2)]-*β*-D-glucopyranoside; (**3**) patuletin 3-*O*-*β*-D-(2″-feruloylglucopyranosyl)(1→6)-[*β*-D-apiofuranosyl(1→2)]-*β*-D-glucopyranoside; (**4**) spinacetin 3-*O*-*β*-D-(2″-*p*-coumaroylglucopyranosyl)(1→6)-[*β*-D-apiofuranosyl(1→2)]-*β*-D-glucopyranoside; (**5**) spinacetin 3-*O*-*β*-D-(2″-feruloylglucopyranosyl)(1→6)-[*β*-D-apiofuranosyl(1→2)]-*β*-D-glucopyranoside; (**6**) spinacetin 3-*O*-*β*-D-glucopyranosyl)(1→6)-*β*-D-glucopyranoside; (**7**) spinacetin 3-*O*-*β* -D-(2″-feruloylglucopyranosyl))(1→6))-*β*-D-glucopyranoside, (**8**) jaceidin 4′-glucuronide; (**9**) 5,3′,4′-trihydroxy-3-methoxy-6:7-methylenedioxyflavone 4′-glucuronide; (**10**) 5,4′-dihydroxy-3,3′-dimetoxy-6:7-methylenedioxyflavone 4′-glucuronide. [b] Trolox equivalents.Solutions of the different compounds were prepared and their activity compared to that of a trolox solution.

**Table 2. Free-Radical Scavenging Activity of Fresh-Cut Spinach, Initially and after 7 Days Storage in Air or MAP at 10 °C**

| | activity[a] |
|---|---|
| initial | 0.56 (0.02) |
| air | 0.28 (0.06) |
| MAP | 0.18 (0.03) |
| AA | 0.94 (0.01) |
| DHAA | 0.00 (0) |

[a] Trolox equivalents. Mean ($n = 3$) (standard deviation).

The effect of MAP on AA, DHAA, and vitamin C was evaluated. Although many studies have reported the total vitamin C content in fresh fruit and vegetables, we investigated both forms of vitamin C, AA, and DHAA. The initial fresh-cut spinach contained AA as a predominant form of vitamin C (Figure 2). However, after 3 days of storage, both in air and MAP, a decrease in AA to one-half of the initial value was noted, followed by a higher reduction after 7 days of storage. No significant differences in the AA content of both air and MAP samples were observed during the storage, in contrast with the results reported by Izumi et al. (1997) who found a higher AA content in low $O_2$ (0.5%, 1%, or 2%) than in air. On the contrary, an accumulation of DHAA was detected, becoming the predominant form of vitamin C during storage. The increase in DHAA was tremendously marked under MAP and resulted the same vitamin C content as the initial samples being maintained.

These results are consistent with those previously reported by Burgheimer et al. (1967), who described that when the $O_2$ concentration in the storage atmosphere is reduced to a low level, a rapid depletion in AA and a simultaneous increase in DHAA occur. However, in our case, although AA disappeared in the same proportion for both storage atmospheres, accumulation of DHAA was higher in MAP than in air.

AA is reversibly oxidized to DHAA in plant tissues (Erdman and Klein, 1982). The natural equilibrium in spinach leaf has been described to be toward a high concentration of AA and low concentration of DHAA. However, this equilibrium was inverted toward DHAA when only small quantities of oxygen were present in the atmosphere, and those low-oxygen concentrations appeared to be reported as causing the change in metabolism (McGill et al., 1966). An increase in the pH of fresh-cut spinach stored in MAP was observed (data not shown), as described for spinach under CA (Burgheimer et al., 1967), and might be responsible for changes in the equilibrium AA−DHAA.

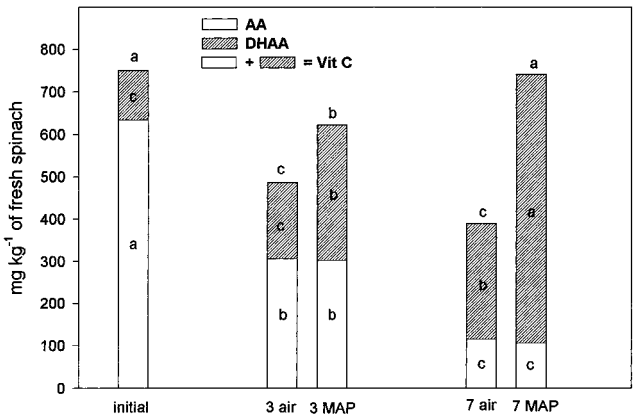


**Figure 2.** Effect of modified atmosphere packaging (MAP) on the AA, DHAA, and vitamin C content of fresh-cut spinach, initially and after 3 and 7 days stored at 10 °C. For each compound (AA, DHAA, and vitamin C), different letters denote significant differences according to Duncan's multiple range test ($P = 0.05$).

Vitamin C (AA + DHAA) was found to disappear rapidly when fresh-cut spinach was stored in air compared to modified atmosphere (Figure 2). However, losses in AA and increases in DHAA-compensated vitamin C and maintained the same content for MAP samples than for initial ones.

The free-radical scavenging activity of the initial and storage samples are shown in Table 2. A decrease in activity was observed, particularly for those samples held under MAP. The free-radical scavenging activity of AA and DHAA was evaluated. The radical scavenging activity of AA was slightly smaller than that of trolox, while this activity completely disappeared when AA was transformed to DHAA. As the antioxidant activity is currently associated with the AA and both air and MAP extracts contained the same AA content, there must be, in addition, some other compounds that confer antioxidant activity. In fact, the most antioxidant flavonoids found in spinach (**1** and **3**) were present in higher amounts in air than in MAP (Table 3).

**Effect of Cooking on Flavonoids and Vitamin C.** After boiling the samples, the cooking water and cooked tissue were analyzed for flavonoid and vitamin C content. The initial flavonoid content was practically divided into two: one-half was dissolved in the cooking water and the other half remained in the cooked tissue (Table 3). After 3 and 7 days of air storage, the total flavonoids detected after cooking remained constant with around 50% of flavonoids in the cooking water and 50% in the cooked tissue. However, the total flavonoids content of the sample stored under MAP decreased significantly after cooking. This decrease was mainly detected in those flavonoids present in the cooking water (Figure 3).

The individual flavonoids were identified and quantified in the cooking water and in the cooked tissue. The

**Table 3. Individual Flavonoids of Fresh-Cut Spinach, Initially and after 7 Days in Air and MAP at 10 °C, and in the Cooking Water and Cooked Tissue after Cooking the Initial Samples[a]**

| | 1 | 2 | 3 | 4 | 5 | 6 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| initial | 92 (1) | 111 (6) | 55 (4) | 19 (3) | 187 (9) | 191 (7) | 143 (3) | 170 (2) | 68 (2) |
| air | 94 (7) | 112 (6) | 66 (8) | 15 (1) | 187 (7) | 221 (16) | 154 (9) | 214 (22) | 87 (8) |
| MAP | 72 (20) | 103 (21) | 56 (15) | 14 (5) | 178 (9) | 201 (11) | 149 (2) | 215 (13) | 89 (8) |
| cooking water | 36 (3) | 35 (9) | 14 (6) | 5 (0) | 78 (6) | 102 (5) | 65 (2) | 84 (1) | 29 (1) |
| cooked tissue | 54 (4) | 74 (7) | 34 (2) | 7 (0) | 122 (3) | 58 (3) | 63 (1) | 45 (3) | 19 (4) |

[a] See Table 1 for flavonoid identification. Values are mg $kg^{-1}$ fresh weight (standard deviation) ($n = 3$).

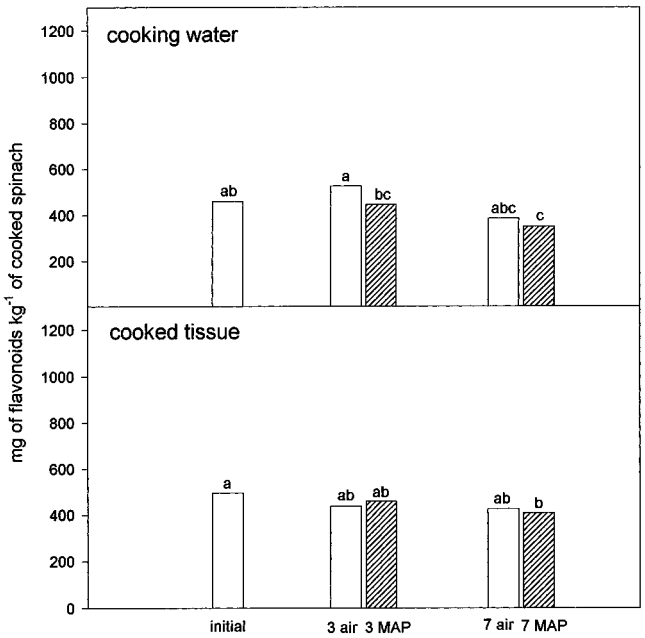


**Figure 3.** Effect of cooking on the flavonoid content of fresh-cut spinach, initially and after 3 and 7 days stored at 10 °C. Different letters denote significant differences according to Duncan's multiple range test ($P = 0.05$).

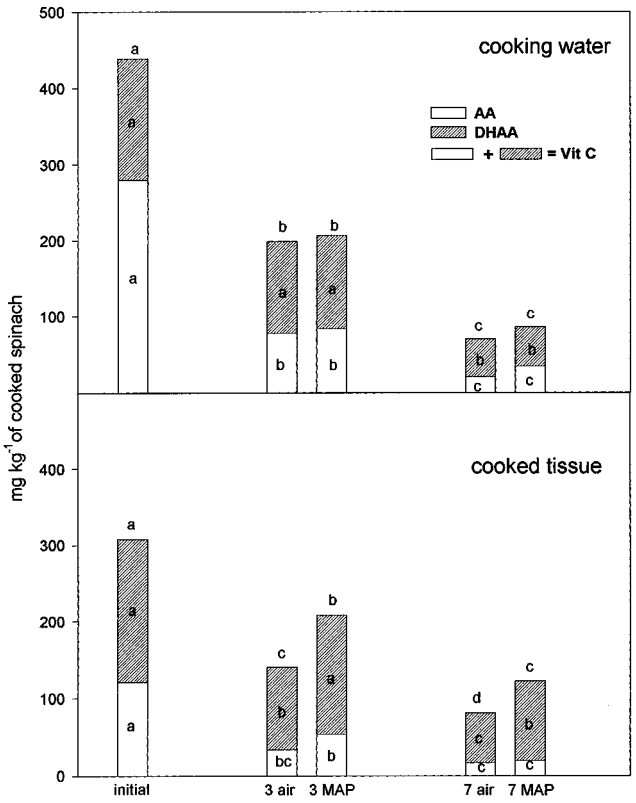


**Figure 4.** Effect of cooking on the AA, DHAA, and vitamin C content of fresh-cut spinach, initially and after 3 and 7 days stored at 10 °C. For each compound (AA, DHAA, and vitamin C) different letters denote significant differences according to Duncan's multiple range test ($P = 0.05$).

effect of cooking on the flavonoids is shown in Table 3. Flavonoid glycosides and acylated derivatives (compounds **1**−**5**) were less extracted from the tissue by the cooking process than the glucuronides derivatives (**6**, **8**−**10**), which were the predominant compounds in the cooking water. In addition, these last compounds were much more degraded due to the cooking process, since a 30% loss was detected when compared to with the original tissue. This could be explained by a higher solubility of glucuronides in water than the glycosides and acylated compounds that are less water soluble and therefore remain in the tissue. It also suggested that components in the tissue are more stable than those in water, where they are more degraded.

Prediction of vitamin C losses was complicated by lack of information about the mechanisms of degradation and the factors that influence them. It has been reported than when large amounts of water are used for cooking, a higher percentage of AA has been found in the cooking liquid, which emphasizes the importance of using minimum amounts of water in vegetable cookery (Erdman and Klein, 1982).

The effect of cooking on AA, DHAA, and vitamin C was evaluated on fresh-cut spinach, initially and after 3 and 7 days of cold storage (Figure 4). Approximately 60% of the initial vitamin C content was extracted in the cooking water, and 40% was recovered in the cooked tissue. This observation was expected to be due to the high vitamin C solubility in water. Initially, AA was the predominant form of vitamin C in the cooking water and DHAA was for the cooked tissue. In both cooking water and cooked tissue, a significant decrease in the AA content was observed after storage. However, there was no significant difference in AA for air and MAP samples. DHAA was more heat stable and there was a significant difference after 3 days when the samples were kept in air. After 7 days, DHAA decreased for air or MAP samples. No statistical differences on AA, DHAA, and vitamin C were detected for air and MAP samples when the cooking water was analyzed, and the same rate of degradation was observed. On the other hand, as Figure 5 shows for the cooked tissue, samples stored 7 days in MAP retained the same vitamin C content as those stored 3 days in air. Therefore, MAP preserved the vitamin C content for the cooked tissue after the storage period.

To study the stability of AA and DHAA to heat, a model solution was assayed. AA and DHAA appeared to be very heat stable and no degradation with temperature was observed in the model solutions. Because of the major losses of vitamin C that occurred after cooking the stored samples, there must be other enzymatic degradation processes different from heat that produce the large vitamin C degradation. However, more re-

search is needed to explain a possible enzymatic oxidation and its relation with pH.

## ABBREVIATIONS USED

AA, ascorbic acid; DHAA, dehydroascorbic acid; OPDA, 1,2-phenylenediamine dihydrochloride; DFQ, 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one; EDTA, ethylenediaminetetraacetic acid.

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