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Biochemical Study of Leaf Browning in Minimally Processed Leaves of Lettuce (*Lactuca sativa* L. Var. *Acephala*)

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A series of biochemical parameters, including the concentration of total ascorbic acid (ASA_{tot}) and the activities of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), and peroxidases (PODs), was investigated during cold storage (72 h at 4 °C in the dark) in fresh-cut (minimally processed) leaves of two lettuce (*Lactuca sativa L.* var. *acephala)* cultivars differing in the susceptibility to tissue browning: Green Salade Bowl (GSB), susceptible, and Red Salade Bowl (RSB), resistant. The two cultivars showed differences also at the biochemical level. The content in ASA_{tot} increased in RSB, as a consequence of increased DHA concentration; conversely, ASA_{tot} diminished in GSB, in which ASA was not detectable after 72 h of storage, thus suggesting a disappearance of ascorbate (both ASA and DHA) into nonactive forms. The antioxidant capacity (as determined by using FRAP analysis) decreased significantly during storage in RSB, while a strong increase was observed in GSB. PAL activity increased soon after processing reaching a maximum by 3 h, then it declined to a relatively constant value in RSB, while in GSB it showed a tendency to decrease in the first few hours from harvest and processing. POD activity, at least for chlorogenic acid, increased significantly during storage only in GSB.

KEYWORDS: Antioxidant ability; ascorbic acid; browning; *Lactuca sativa*; minimal processing; peroxidases; phenylalanine ammonia lyase; polyphenol oxidase

INTRODUCTION

Fresh-cut fruits and vegetables have generally a short shelf life and may show tissue browning as a consequence of stressful conditions that occur during post-harvest processing (handling, cutting, packaging, etc.) and storage (*1*). Indeed, tissue browning is a typical disorder of fresh-cut lettuce (*2*).

Browning is considered to result from an increase in enzymatic activities associated with the loss of cell integrity and compartmenting that is provoked principally by cutting (*3*, *4*). Current biochemical model for plant tissue browning considers the metabolism of phenylpropanoids. In response to cutting, phenylalanine ammonia lyase (PAL, EC 4.3.1.5), the committed enzyme in the phenylpropanoid pathway, produces phenols; these compounds are then oxidized by the action of polyphenol oxidase (PPO, EC 1.10.3.1) and peroxidase (POD, EC 1.11.1.7) (*1*) to quinones, which in turn spontaneously polymerize to form brown pigments.

The activity of PAL is low in nonstressed plants, but it increases in the presence of a stress, such as wounding (e.g., *5*, *6*). Moreover, PAL activity may be an index of the extent to which fresh-cut products are damaged during processing, then of their shelf life and quality, especially of color and texture (*3*).

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There are many evidences about the involvement of PPO (*7*) and POD (*8*) in tissue browning of fresh-cut products. PPO, a copper-containing enzyme widely distributed in plants, catalyzes two different reactions in the presence of oxygen (*7*): (i) the hydroxylation of monophenols to *o*-diphenols; and (ii) the oxidation of *o*-diphenols to *o*-quinones. POD, another enzyme almost ubiquitous in plants, catalyzes many reactions, and its activity may be modulated by both biotic and abiotic stresses, including wounding (*8*). In the presence of small amounts of hydrogen peroxide, POD can oxidize both mono- and diphenols (*8*). PODs are also active in the H_2O_2-dependent polymerization of hydroxycinnamyl alcohols (i.e., monolignols) in lignin biosynthesis (*9*).

Ascorbic acid (ASA) may represent a marker compound for the extent of oxidation in fresh-cut vegetables (*10*), although it is destroyed during processing (*11*). It is known that ASA inhibits the activity of PPO because it reduces the enzymatically formed *o*-quinones to their precursors diphenols, thus reducing the severity of browning (*12*, *13*); in this process, dehydroascorbate (DHA) is formed. For its antioxidant properties, ASA is used to delay cut-edge browning in minimally processed products (*14*). On the other hand, it is known that the browning of citrus juice is associated with the oxidation of ASA to DHA, which is degraded to brown pigments (*15*). The current literature reported very few data on the relationship between ASA metabolism and the occurrence of browning in fresh-cut lettuce leaves. We are aware only of the paper published by Cantos et

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al. (*16*), who did not find any relationship between the incidence of browning and DHA level in six different cultivars of lettuce.

The aim of the present study was to characterize the change of ascorbate content and the activities of PAL, PPO, and POD that occurred upon storage in minimally processed leaves of two lettuce (*Lactuca sativa* var. *acephala*) cultivars, which in a preliminary experiment showed different susceptibility to browning: Green Salade Bowl (GSB), susceptible, and Red Salade Bowl (RSB), resistant. The terms "red" and "green" do not refer to actual differences in leaf color in that (very early; three weeks from sowing) stage of plant development.

MATERIALS AND METHODS

Plant Material. Lettuce seeds were sown in perlite and then grown in a glasshouse in stagnant nutrient solution aerated with compressed air (floating system). The composition of the nutrient solution was the following (mmol L^{-1}): 14 N, 1.25 P, 10.7 K, 5.0 Ca, 1.6 Mg, 0.47 S, 0.014 Mn, 0.004 Cu, 0.005 Zn, 0.01 B, and 0.002 Mo. The pH of the solution was 5.7 ± 0.3, and electrical conductivity was 2.6 ± 0.2 mS cm^{-1}. All experiments were carried out in spring and early summer of 2004; minimum night temperature was 14−18 °C, maximum day temperature ranged from 28 to 32 °C, while minimum air relative humidity was around 50−60%.

At the harvest, the leaves were washed with chlorinated drinking water and centrifuged with a manual salad spinner to remove excess water. Afterward, leaves were cut (approximately 1 cm × 2 cm) perpendicular to the midrib with stainless steel scissors. Samples of approximately 150 g of leaf cuts were placed in sealed plastic containers (1.500 L) in water-saturating atmosphere at 4 °C in the dark.

Chlorophyll fluorescence and visual symptoms of browning were measured daily. The activity of selected enzymes, the antioxidant capability, and ASA/DHA content were analyzed in cut leaves both prior to and after storage; PAL was assayed also during the first hours after processing. All analyses were carried out on leaf lamina.

Visual Symptoms. Tissues were scored to estimate visually the extent of browning development. The rating scale reported by Ke and Saltveit (*17*) was used to estimate visually the extent of browning in lettuce cuts: the score ranged from 0 (no injury) to 9 (severe injury, i.e., complete browning of cut).

Chlorophyll Fluorescence. The ratio of variable to maximal chlorophyll fluorescence ($F_v/F_m = [(F_m - F_0)/F_m]$) of the leaf lamina was measured using an amplitude-modulated fluorometer (PAM-2000, Heinz Walz, Germany) after 40 min of dark adaptation (*18*). Saturating light was about 8000 μmol m^{-2} s^{-2} with a duration time of 800 ms.

Phenolic Compounds. Tissue concentration of phenol compounds was determined according to Ke and Saltveit (*6*). A 10-g sample of leaf tissue was homogenized with 20 mL of HPLC grade methanol, and the homogenates were filtered and centrifuged at 15000*g* at 20 °C for 15 min. Then, the absorbance of an aliquot of the supernatant was read at 320 nm.

Antioxidant Capability. Ferric-reducing antioxidant power (FRAP) assay was based upon the methodology of Benzie and Strein (*19*). The assay was carried out with newly prepared FRAP reagent, which contained 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate (pH 3.6). For this analysis, a 0.1-mL aliquot of the methanol extract was added to 0.9 mL of FRAP reagent and mixed; after the mixture was kept at 20 °C for 4 min, the absorbance at 593 nm was determined against a water blank. Calibration was against a standard curve (50−1000 μM ferrous ion) produced by the addition of freshly prepared ammonium ferrous sulfate. Antioxidant capability was expressed as mmol Fe^{2+}/100 g fresh weight.

Enzyme Assay. PAL activity was measured as described by Ke and Saltveit (*5*), with some modification. A 2-g sample of tissue was homogenized at 4 °C with 16 mL of 50 mM borate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol and 0.2 g of poly(vinylpyrrolidone). The homogenate was filtrated through 4 layers of cheesecloth and centrifuged at 20000*g* at 4 °C for 20 min. The supernatant was assayed for PAL activity after the addition of 0.55 mL of 50 mM l-phenylalanine and incubation at 40 °C for 1 h. The absorbance was measured at 290 nm before and after incubation. One unit of PAL activity equals the amount of PAL that produced 1 μmol of *trans*-cinnamic acid in 1 h; it is expressed as μmol g^{-1} FW h^{-1}.

PPO enzymes was extracted as described by Loiza-Velarde et al. (*2*). A 4-g sample of tissue was homogenized at 4 °C with 12 mL of 50 mM phosphate buffer (pH 6.5). The homogenate was filtered and centrifuged at 20000*g* at 4 °C for 20 min. PPO activity was assayed as reported by Couture et al. (*3*). A 0.5-mL volume of enzyme extract was incubated with 0.2 mL of 0.1 mM caffeic acid ethanolic solution, and the absorbance was recorded at 480 nm over a period of 5 min. One unit of PPO activity was defined as the amount of enzyme which causes a change of 0.01 in absorbance per minute.

Peroxidases (POD) were extracted as previously described for PPO. The assays of all POD were done in 1-mL volumes at 25 °C (*20*). The absorbance was recorded at selected wavelengths, depending on the substrate. For measurement of guaiacol peroxidase activity (A_{470}), the assay contained 800 μL of 10 mM guaiacol in 50 mM potassium phosphate buffer, pH 6, 80 μL of extract and 20 μL of distilled water, and 100 μL of 35 mM H_2O_2. TMB (tetramethylbenzidine) peroxidase activity (A_{654}) was measured with minor modifications to the method of Imberty et al. (*21*). The assay contained 800 μL of phosphate−citric acid buffer (90 mM Na_2HPO_4 and 55 mM citric acid), pH 4.5, 50 μL of extract, and 100 μL of 35 mM H_2O_2. Assay of chlorogenic acid peroxidase (A_{410}) and caffeic acid peroxidase (A_{450}) were conducted essentially as described by Mäder et al. (*22*). The chlorogenic acid peroxidase assay contained 800 μL of 50 mM potassium phosphate buffer, pH 6.5, 50 μL of 80 mM chlorogenic acid, 50 μL of extract, and 100 μL of 35 mM H_2O_2. The caffeic acid peroxidase assay contained 800 μL of McIlvaine (114 mM Na_2HPO_4 and 43 mM citric acid), pH 5.5, 50 μL of 80 mM caffeic acid, 50 μL of extract, and 100 μL of 35 mM H_2O_2. In all cases, POD assays were initiated by the addition of H_2O_2. The activities of PODs are expressed as ΔA_λ min^{-1} g^{-1} fresh weight.

Total soluble protein was determined according to the method of Lowry et al. (*23*) with bovine serum albumin as a standard.

Ascorbic Acid Content. ASA and DHA were determined spectrophotometrically as described by Kampfenkel et al. (*24*). Leaf material (about 0.4 g) was transferred to 6% (w/v) trichloroacetic acid (TCA) and the mixture was stirred continuously for 15 min; the mixture was centrifuged at 15000*g* for 5 min at 4 °C, and the supernatant was immediately used for analysis of ascorbate.

Statistical Treatments. The experiment was repeated three times with similar results; the paper reports the results from representative experiments. Mean values of three replicates were compared using the LSD (least significant difference) test after two-way analysis of variance (ANOVA) with storage time and cultivar as variability factors.

RESULTS AND DISCUSSION

As expected, the two cultivars of lettuce were characterized by a different susceptibility to browning. Indeed, only GSB showed evident browning during storage: the score in GSB was 0.6 after 48 h of cold storage, then increased significantly, reaching a value of 3.9 by 72 h. The score in RSB was always nil, and the leaves remained green up to 72 h and even longer. Indeed, in an independent experiment, RSB leaves did not exhibit any important browning symptoms up to 10 days from packaging. Also considering that in Italian markets fresh-cut products are typically consumed within 3−5 days after harvest and processing, the biochemical investigations were restricted to samples taken after 72 h, when the differences in browning susceptibility between selected lettuce cultivars were noteworthy.

The F_v/F_m ratio is the index of the PSII photochemical efficiency, and in healthy leaves it ranges between 0.80 and 0.85 (*25*). No significant differences in the F_v/F_m ratio upon storage in the two cultivars of lettuce were found **(Table 1)**. This suggests that no alteration affected the photosynthetic process and the leaves remained viable.

Table 1. F_v/F_m Ratio Measured in Fresh-Cut Leaves of *Lactuca sativa* Var. *Acephala* Cv. Red Salade Bowl (RSB) and Cv. Green Salade Bowl (GSB) Stored at 4 °C in the Dark [a,b]

	storage			
	0 h	24 h	48 h	72 h
RSB	0.845 ± 0.002 a	0.846 ± 0.006 a	0.840 ± 0.003 a	0.836 ± 0.005 a
GSB	0.838 ± 0.011 a	0.835 ± 0.004 a	0.841 ± 0.003 a	0.835 ± 0.005 a

[a] Each value represents the mean of three replicates (±SD); the means followed by the same letters are not significantly different at the 5% level. [b] Ratios were determined at 25 °C after 40 min in the dark.

Table 2. Content of Phenolic Compounds (ABS_{320} g^{-1} FW) and Antioxidant Capacity (FRAP; mmol Fe^{2+}/100 g FW) in the Fresh-Cut Leaves of *Lactuca sativa* Var. *Acephala* Cv. Red Salade Bowl (RSB) and Cv. Green Salade Bowl (GSB) Stored at 4 °C in the Dark[a]

	storage	
	0 h	72 h
phenolic compounds		
RSB	0.030 ± 0.004 a	0.033 ± 0.005 a
GSB	0.037 ± 0.006 a	0.046 ± 0.008 a
FRAP		
RSB	2.04 ± 0.38 b	1.50 ± 0.09 c
GSB	0.75 ± 0.05 d	4.04 ± 0.01 a

[a] Each value represents the mean of three replicates (±SD); the means followed by the same letters are not significantly different at the 5% level.

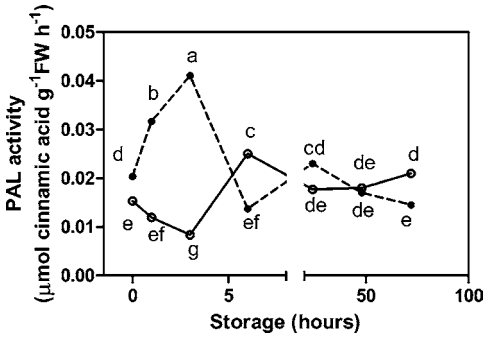

Figure 1. Phenylalanine ammonia lyase (PAL) activity in the fresh-cut leaves of *Lactuca sativa* var. *acephala* cv. Red Salade Bowl (RSB; closed circle) and cv. Green Salade Bowl (GSB; open circle) stored at 4 °C in the dark. The activity was determined at harvest (hour 0) and at different times up to 72 h. Each value represents the mean of three replicates (±SD); the means followed by the same letters are not significantly different at the 5% level.

In two cultivars of lettuce (Iceberg and Romaine), Kang and Saltveit (*26*) observed a significant linear correlation between the antioxidant capacity (as determined by FRAP assay) and the phenolic content that was associated with tissue browning. Such a correlation was not found in our study, however. Indeed, the amount of phenolic compounds did not changed significantly during storage in both cultivars (**Table 2**). By contrast, the antioxidant capability (as determined with FRAP assay, as in the study conducted by Kang and Saltveit) changed significantly upon storage, although in a different way in the two cultivars (**Table 2**); in fact, FRAP increased significantly in GSB (up to 4.04 mmol Fe^{2+}/100 g) and decreased in RSB.

Minimal processing resulted in a typical induction pattern of PAL activity in RSB leaf cuts (**Figure 1**). PAL activity increased soon after processing and reached a maximum after 3 h, then it declined and remained relatively constant up to 72 h. The kinetics of induction was similar to those reported for Iceberg

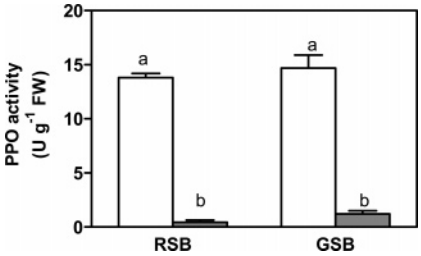

Figure 2. Polyphenol oxidase (PPO) activity in the fresh-cut leaves of *Lactuca sativa* var. *acephala* cv. Red Salade Bowl (RSB) and cv. Green Salade Bowl (GSB) stored at 4 °C in the dark. The activity was determined at harvest (white bar) and 72 h later (gray bar). Each value represents the mean of three replicates (±SD); the means followed by the same letters are not significantly different at the 5% level.

lettuce (e.g., *6*, *27*). The decrease observed in RSB tissues in PAL activity may have resulted from the presence of inactivating factor, as suggested by Ritenour and Saltveit (*28*), and not from reduced protein synthesis, since the protein content of leaf tissues did not changed significantly upon storage in both lettuce genotypes (data not shown).

A transient increase in PAL activity was observed also in GSB leaves, albeit with some delay compared to RSB (**Figure 1**); in GSB, PAL activity showed a tendency to decrease in the first few hours of storage. In lettuce, Campos-Vargas and Saltveit (*29*) reported a rise in PAL activity from 8 to 24 h after wounding, while Campo-Vargas et al. (*27*) observed a lag time of 6 with the maximum recorded at 24 h.

The activity of PPO was similar in GSB and RSB and decreased markedly upon storage (**Figure 2**). Cantos et al. (*16*) reported that wounding led to an exponential increase of PPO activity in lettuce leaves due to the activation of a latent form of PPO by newly formed proteases (*30*). On the other hand, other authors reported no significant changes in PPO activity in Iceberg lettuce leaf cuts during cold storage (*6*, *31*). However, Cantos et al. (*16*) did not observe a clear correlation between browning and PPO activity.

The involvement of POD in enzymatic browning of fresh-cut products has been questioned mainly because of the low H_2O_2 content in vegetables tissues (*32*). However, the possible role of POD in enzymatic browning through a synergistic effect PPO−POD is reported by Richard-Forget and Gauillard (*33*). Since POD can utilize many different substrates (*20*), in this work POD activity was assayed in the presence of both natural (caffeic and chlorogenic acid) and artificial (guaiacol and TMB) hydrogen donors. Guaiacol, TMB, and caffeic acid POD activities did not vary substantially during storage in both cultivars (**Figure 3A−C**); a significant, albeit slight, increase in caffeic acid POD was found only in RSB. On the contrary, during storage, chlorogenic acid POD increased by 1 order of magnitude in the susceptible cultivar GSB, while no significant changes were observed in RSB (**Figure 3D**). Both caffeic and chlorogenic acids have been reported as abundant phenolic compounds in lettuce leaves (*34*); therefore, a noticeable difference in POD activities for one or both of these compounds may be related to differences in the susceptibility to browning. Ke and Saltveit (*6*) and Cantos et al. (*16*) reported that wounding led to increased POD activity in lettuce leaves during storage, probably due to de novo synthesis of peroxidase isoenzymes.

The content in total ASA (ASA_{tot}) increased significantly in RSB, due to a rise in DHA (**Figure 4C**). Similar results were reported by Barry-Ryan and O'Beirne (*11*), who found that in Iceberg lettuce a large amount of ASA was converted into DHA, especially in open bags, due to the larger availability of oxygen

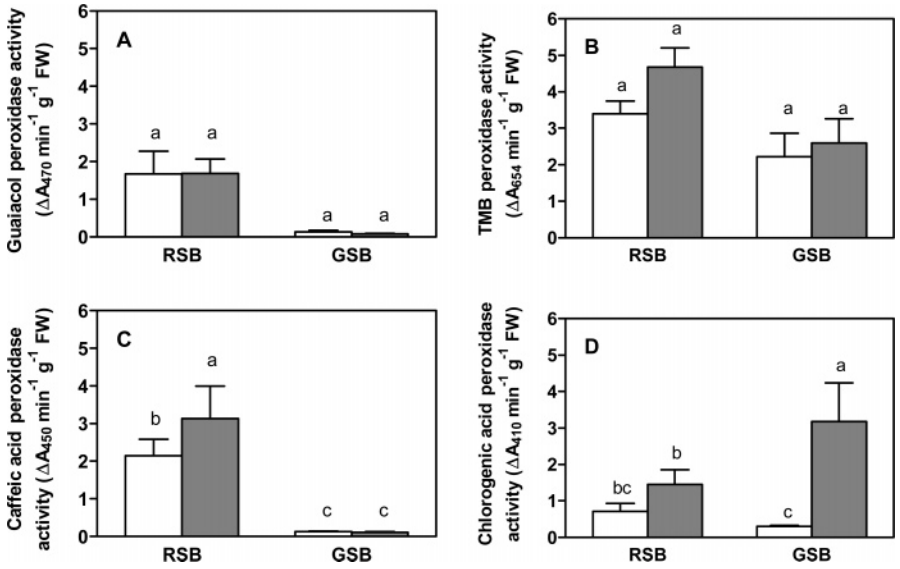

Figure 3. Peroxidase (POD) activity in the fresh-cut leaves of *Lactuca sativa* var. *acephala* cv. Red Salade Bowl (RSB) and cv. Green Salade Bowl (GSB) stored at 4 °C in the dark. POD activity was determined by using different substrates: (**A**) guaiacol; (**B**) TMB; (**C**) caffeic acid; (**D**) chlorogenic acid. The activity was determined at harvest (white bar) and 72 h later (gray bar). Each value represents the mean of three replicates (±SD); the means followed by the same letters are not significantly different at the 5% level.

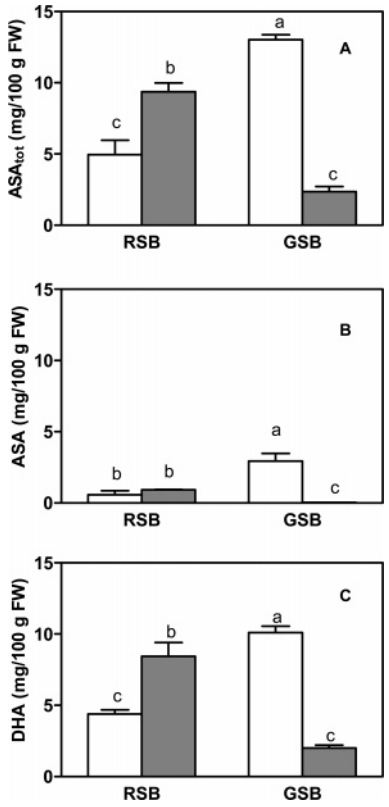

Figure 4. Total ascorbic acid (ASA_{tot}; **A**), ascorbic acid (ASA; **B**), and dehydroascorbic acid (DHA; **C**) contents in fresh-cut leaves of *Lactuca sativa* var. *acephala* cv. Red Salade Bowl (RSB) and cv. Green Salade Bowl (GSB) stored at 4 °C in the dark. The analyses were carried out at harvest (white bar) and 72 h later (gray bar). Each value represents the mean of three replicates (±SD); the means followed by the same letters are not significantly different at the 5% level.

there as compared to that in sealed packages. An increase in DHA during storage was found also by Petersen and Berends (*35*) in green sweet peppers.

By contrast, ASA_{tot} diminished in GSB, in which ASA was not detectable after 72 h of storage (**Figure 4A**,**B**). This suggests a disappearance of ascorbate (both ASA and DHA) into nonactive forms in the susceptible cultivar GSB. As already reported in the Introduction section, the browning of citrus juice is caused primarily by the degradation of DHA that produces brown pigments (*15*).

In conclusion, the results obtained in this study conducted with fresh-cut leaves of two lettuce cultivars suggest the possible involvement of ASA metabolism in the occurrence of tissue browning, in contrast with the conclusions reported by Cantos et al. (*16*). Indeed, it was found that in the sensitive cultivar GSB the ASA_{tot} decreased markedly during storage, presumably because of a conversion of ascorbate to nonactive forms. Moreover, in GSB a marked increase in the activity of POD (at least for chlorogenic acid) was also found, which may be responsible for enhanced synthesis of *o*-quinones that are converted to brown pigments.

Further work is now in progress to investigate the interaction between ASA metabolism and browning in fresh-cut lettuce, also in light of chemical characterization of brown pigments that accumulate in damaged leaves.

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