Phenolic Metabolites in Red Pigmented Lettuce (*Lactuca sativa*). Changes with Minimal Processing and Cold Storage

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Minimally processed products are more perishable than unprocessed raw materials and readily deteriorate in quality, especially in color and texture. The effects of minimal processing on phenolic metabolites have been studied on red lettuce. Three different tissues of cv. Lollo Rosso were separated as white, green, and red and stored at 5 °C for 7–14 days. The phenolic acids caffeoyltartaric, chlorogenic, dicaffeoyltartaric, and dicaffeoylquinic, the flavonoids quercetin 3-glucuronide, quercetin 3-glucoside, quercetin 3-(6-malonylglucoside), and the new compound quercetin 3-(6-malonylglucoside) 7-glucoside, and the anthocyanin cyanidin 3-malonylglucoside were isolated and identified in lettuce extracts. Phenolic compound content was evaluated immediately after cutting and after storage of the minimally processed products at 5 °C. In midrib, wounding induced an increase in phenylpropanoids during storage, particularly phenolic acids and anthocyanins. On the contrary, wounding did not induce significant changes in the caffeic acid derivatives and flavonoids in green and red tissues. The anthocyanin content decreased in both green and red tissues during storage.

Keywords: *Lettuce; Lactuca sativa; Lollo Rosso; anthocyanins; phenolics; flavonoids; minimal processing; wound-induced changes; pigment stability; quality*

INTRODUCTION

The use of minimally processed lettuce (*Lactuca sativa* L.) has continued to increase in salad bars and fast food because of its long storage life and good quality. However, one of the major causes of quality loss is still the discoloration of the cut pieces. Phenolic metabolites are important since they are involved in tissue browning by the action of polyphenol oxidase (PPO) (EC 1.10.3.1) (Sharples et al., 1963). In addition, they have nutritional relevance, due to their antioxidant and free-radical-scavenging properties (Manach et al., 1996).

When lettuce is processed, the tissue is wounded and an increase in the concentration of soluble phenolic compounds has been reported in the midribs of iceberg lettuce (Ke and Saltveit, 1989) and other lettuce cultivars including butter leaf and romaine lettuce (Tomás-Barberán et al., 1997a) and in lettuce stem disks (Tomás-Barberán et al., 1997b). However, the effect of wounding on phenolic metabolite composition of pigmented tissues (green photosynthetic tissue and red pigmented tissue) has never beed studied.

Although iceberg lettuce is predominantly used for prepared salads, other types of lettuce are now used in salad mixes (López-Gálvez et al., 1996). Red lettuce has become popular due to the pigmentation of the tissues as one of the main quality characteristics. However, there is not much information on red lettuce about the anthocyanin (Yamaguchi et al., 1996) and other phenolics content (Wöldecke and Herrmann, 1974; Winter and Herrmann, 1986) and their stability after minimal processing. The purpose of this work was to identify the phenolic metabolites in red lettuce cv. Lollo Rosso and the changes with minimal processing and cold storage.

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EXPERIMENTAL PROCEDURES

Lettuce. Commercially grown red lettuce (cv. Lollo Rosso) was harvested at optimal maturity stage in November 1996 and February 1997 in Torrepacheco (Agricola Mar Menor Coop., Murcia, Spain). Lettuce heads were transported to the laboratory and stored at 2 °C until processed the next day.

Extraction of Phenolics for Isolation and Identification. Red lettuce was shredded with a sharp stainless steel knife in uniform pieces 1 cm thick. Fresh material was lyophilized and ground in an electric blender. Eighty grams of ground lettuce was macerated statically in a beaker with MeOH/AcOH/H_2O (25:4:21) (700 mL) for 24 h at 20 °C (this was carried out three times to extract all phenolic compounds). The three extracts were combined and vacuum-filtered through filter paper (Whatman No. 1) and concentrated under reduced pressure (40 °C) until all of the methanol had been evaporated. With this concentrate a batch was prepared with nonionic polymeric resin Amberlite XAD-2 (Sigma, St. Louis, MO) (column of 50 × 4 cm). The batch was stirred (20 °C) with a magnetic stirrer for 1 h to allow the adsorption of phenolic compounds on the resin particles. This procedure recovers >95% of the phenolics present in aqueous solutions (Tomás-Barberán et al., 1992). The column was then packed with the wet resin and washed with distilled water to remove all water soluble substances, and the phenolic compounds were eluted with methanol (flow rate = 20 mL min^{-1}). The methanol extract was then concentrated to dryness under reduced pressure (40 °C) and redissolved in 10 mL of MeOH/AcOH/H_2O (25:4:21).

Isolation of Compounds. The extract recovered from the Amberlite XAD-2 was chromatographed on a Sephadex LH-20 (Pharmacia, Upssala, Sweden) column (45 × 3 cm) equilibrated with MeOH/AcOH/H_2O (25:4:21), and the different phenolic fractions were visualized under UV light (254 and 360 nm) and daylight (anthocyanin detection). Elution was performed at a solvent flow rate of 2 mL min^{-1}. The fractions containing acids, flavonoids, and anthocyanins were then separated by reversed-phase low-pressure liquid chromatography (LPLC) using a Lobar column (C_{18}, 44 × 3.7 cm) (Merck, Darmstadt, Germany) and eluting with methanol in different proportions (in water plus 5% HOAc) with a solvent flow rate of 3 mL min^{-1}. The composition of the fractions obtained was checked by analytical HPLC (see below), and the different

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compounds were purified by semipreparative HPLC on a Spherisorb ODS-2 column (25 × 0.7 cm, 5 μm particle size), using an acid solvent with an isocratic flow of 2 mL min^{-1}.

UV−Vis. The spectra of the different isolated compounds were recorded with a Secoman Anthelie Graphics spectrophotometer. The phenolic acids and flavonoids dissolved in methanol and the anthocyanin in methanol plus 2% HCl (v/v). The spectra of the flavonoids were also recorded after addition of the classical shift reagents (Mabry et al., 1970).

Hydrolytical Procedures. Acid and alkaline hydrolyses were achieved as described previously Ferreres et al. (1996), and enzyme hydrolysis was performed according to the method of Ferreres et al. (1997).

Paper Electrophoresis. This was achieved according to method of Harborne and Boardley (1985) to detect the presence of acylation with organic dicarboxylic acids.

^{1}H NMR (300 MHz, DMSO-d_6): (**1**) δ 7.54 (1H, d, J = 16.2 Hz, H-β caffeoyl), 7.05 (1H, s, H-2), 7.02 (1H, d, J = 9 Hz, H-6), 6.78 (1H, d, J = 9 Hz, H-5), 6.24 (1H, d, J = 16.2 Hz, H-α caffeoyl), 5.30 (1H, d, J = 3 Hz, HC-O-caff in tartaric acid residue), 4.50 (1H, d, J = 3 Hz, HC-OH in tartaric acid residue); (**2**) 7.43 (1H, d, J = 18 Hz, H-β), 7.00 (1H, d, J = 2 Hz, H-2 caffeoyl), 6.97 (1H, dd, J = 2 and 9 Hz, H-6 caffeoyl), 6.77 (1H, d, J = 9 Hz, H-5 caffeoyl), 6.15 (1H, d, J = 18 Hz, H-α), 5.08 (1H, m, H-5 quinic), 3.93 (1H, m, H-3 quinic), 3.56 (1H, dd, H-4 quinic acid), 1.8−2.2 (4H, m, H-2 and H-6 quinic acid); (**3**) 7.54 (2H, d, J = 18 Hz, H-β), 7.08 (2H, s, H-2), 7.05 (2H, d, J = 9 Hz, H-6), 6.77 (2H, d, J = 9 Hz, H-5), 6.33 (2H, d, J = 18 Hz, H-α), 5.62 (2H, s, HC-O-caffeoyl in tartaric residue); (**5**) 7.48 (1H, d, J = 18 Hz, H-β caffeoyl linked in 3-position of quinic acid), 7.44 (1H, d, J = 18 Hz, H-β caffeoyl linked in 5-position of quinic acid), 7.06 (1H, d, J = 2 Hz, H-2 caffeoyl linked in 3-position of quinic acid), 7.04 (1H, d, J = 2 Hz, H-2 caffeoyl linked in 5-position of quinic acid), 6.97 (2H, dd, J = 2 and 9 Hz, H-6 caffeoyl in 3- and 5-positions of quinic acid), 6.72 (2H, d, J = 9 Hz, H-5 caffeoyl in 3- and 5-positions of quinic acid), 5.30 (1H, m, H-3 quinic acid), 5.18 (1H, m, H-5 quinic acid), 3.75 (1H, m, H-4 quinic acid), 1.8−2.3 (4H, m, H-2 and H-6 quinic acid); (**4**) 7.55 (1H, d, J = 2 Hz, H-2′), 7.52 (1H, dd, J = 2 and 9 Hz, H-6′), 6.85 (1H, d, J = 9 Hz, H-5′), 6.75 (1H, d, J = 2 Hz, H-8), 6.44 (1H, d, J = 2 Hz, H-6), 5.41 (1H, d, J = 7.5 Hz, H-1 glucose 3-position), 5.08 (1H, d, J = 7.5 Hz, H-1 glucose 7-position), 4.20 (1H, dd, J = 11 and 1.5 Hz, H-6_A of the glucose in 3-position), 4.05 (1H, dd, J = 11 and 5 Hz, H-6_B of the glucose in 3-position), 3.12 (2H, s, CH_2 malonyl); (**9**) 7.52 (1H, d, J = 2 Hz, H-2′), 7.49 (1H, dd, J = 2 and 9Hz, H-6′), 6.84 (1H, d, J = 9 Hz, H-5′), 6.40 (1H, d, J = 2 Hz, H-8), 6.20 (1H, d, J = 2 Hz, H-6), 5.39 (1H, d, J = 8 Hz, H-1 glucose), 4.21 (1H, dd, J = 11 and 1.5 Hz, H-6_A of glucose), 4.03 (1H, dd, J = 11 and 5 Hz, H-6_B of glucose), 3.10 (2H, s, CH_2 malonyl).

^{13}C NMR (60 MHz, DMSO-d_6): (**9**) δ 177.3 (C4), 167.7 (CO malonyl), 166.5 (CO malonyl), 164.1 (C7), 161.2 (C5), 156.3 (C2 and C9), 148.4 (C-4′), 144.7 (C-3′), 133.1 (C-3), 121.5 (C-1′), 121.0 (C-6′), 116.1 (C-5′), 115.1 (C-2′), 103.9 (C-10), 101.0 (C-1 glucose), 98.7 (C-6), 93.5 (C-8), 76.1 (C-3 glucose), 73.9 (C-2 and C-5 glucose), 69.5 (C-4 glucose), 63.6 (C-6 glucose), 41.0 (CH_2 malonyl).

FAB-MS. These were achieved in the positive mode using *m*-nitrobenzyl alcohol matrix as reported previously (Ferreres et al., 1996), with addition of trifluoroacetic acid in the analysis of the anthocyanin.

Minimal Processing and Storage. Lettuce external leaves were removed, and the next uninjured leaves were carefully excised. Three different tissues were separated: white, green, and red tissues. Sections were cut into 2 × 2 cm pieces with a blade to simulate the industrial process. Midribs were selected from the lower third of the leaf, while sections of green tissue were from the middle of the leaf and sections of red tissue from the top. Excised tissues were dipped in a 50 ppm chlorine bath for 1 min at 4 °C and then centrifuged to remove surface moisture. All of the steps were conducted at 4 °C to control enzymatic reactions. Samples of 50 g were placed in perforated plastic bags and held in cells flushed with humidified air (95% relative humidity). Three replicates per tissue were stored for 7 and 14 days at 5 °C. Initial and stored samples were stored at −80 °C for phenolic analysis.

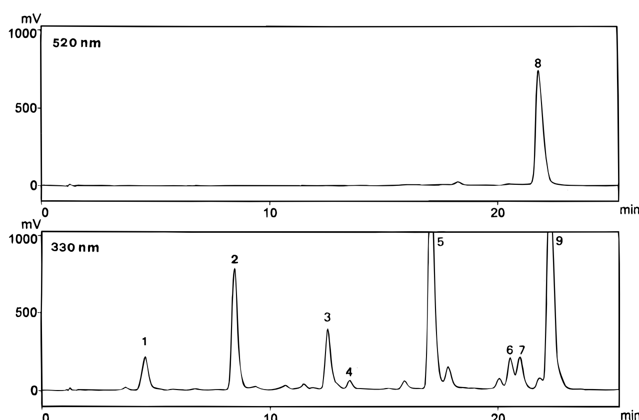

Figure 1. Chromatogram of Lollo Rosso lettuce extracts: **1**, caffeoyltartaric; **2**, chlorogenic acid; **3**, dicaffeoyltartaric acid; **4**, quercetin 7-glucoside 3-(6″-malonylglucoside); **5**, dicaffeoylquinic acid; **6**, quercetin 3-glucuronide; **7**, quercetin 3-glucoside; **8**, cyanidin 3-malonylglucoside; **9**, quercetin 3-malonylglucoside.

Sample Preparation for Quantitative Analysis. Five grams of frozen material was homogenized with 5 mL of MeOH/AcOH/H_2O (25:4:21) for 1 min with an Ultra-turrax homogenizer at 24 000 rpm, and the homogenate was filtered through four layers of cheesecloth. The extracts were filtered through a 0.45 μm before HPLC analysis.

HPLC Analyses. These were done with a Merck-Hitachi gradient liquid chromatograph with a Model L-3000 pump and a Shimadzu 6200 diode array detector. Separations were achieved on a Licrochart column (Merck) (RP-18, 12 × 0.4 cm; 5 μm particle size), using as mobile phases water/formic acid (19:1 v/v) (A) and methanol/formic acid (19:1 v/v) (B). The solvent flow rate was 1 mL min^{-1}, and a linear gradient was used starting with 5% B in A to reach 40% B in A in 25 min. Samples of 20 μL were injected and soluble phenolic compounds were separated. Chromatograms were recorded at 330 nm for phenolic acid and flavonoid quantification and at 520 nm for anthocyanins. Phenolic acids were quantified as chlorogenic acid (5-caffeoylquinic acid) using an authentic marker (Sigma), flavonols as quercetin 3-malonylglucoside (compound **9**), and anthocyanins as cyanidin 3-malonylglucoside (compound **8**). The results were expressed as micrograms per gram of fresh weight.

Statistical Analyses. All data were the mean of three replicates and were tested by analysis of variance procedures with significances of 95%.

RESULTS AND DISCUSSION

Isolation and Identification of Phenolic Acids, Flavonoids, and Anthocyanins. Four main caffeic acid derivatives (**1**−**3** and **5**), four flavonols (**4**, **6**, **7**, and **9**), and one anthocyanin (**8**) were isolated from the leaves of fresh harvested red lettuce (Lollo Rosso) extracts (Figure 1).

Phenolic Acids. Compounds **1**−**3** and **5** showed identical UV spectra, as caffeic acid derivatives. After acid hydrolysis, all yielded caffeic acid; **1** and **3** yield tartaric acid and **2** and **5** quinic acid. ^{1}H NMR spectra of these compounds were recorded, and compounds **1** and **3** showed one and two caffeoyl residues, respectively, linked to the hydroxyls of tartaric acid. Dicaffeoyltartaric acid was a symmetrical molecule, and, therefore, only one singlet was observed for the two CH protons of tartaric acid (singlet at 5.62 ppm). On the contrary, caffeoyltartaric acid showed two doublets with an integration of one proton corresponding to different

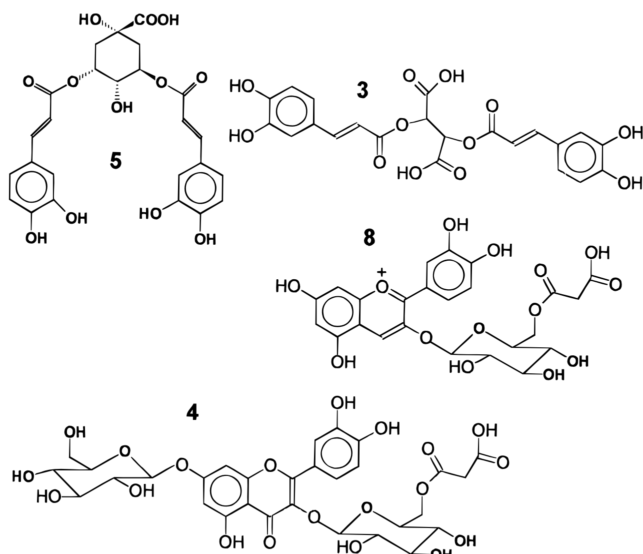

Figure 2. Structures of the isolated phenolics.

CH groups, one linked to a free hydroxyl (4.50 ppm), and another one in which the hydroxyl was esterified with caffeic acid (5.30 ppm). Thus, compound **1** was identified as caffeoyltartaric acid and compound **3** as dicaffeoyltartaric acid (Figure 2).

Compounds **2** and **5** had one and two caffeoyl residues, respectively, by one molecule of quinic acid. The occurrence of quinic acid was confirmed by FAB-MS of compound **2** and by the ^{1}H NMR data of both compounds. Compound **2** coincided both chromatographically and spectroscopically with an authentic marker of chlorogenic acid (5-caffeoylquinic acid). Compound **5** was similar to **2**, but in this case an additional caffeic acid residue was linked to the quinic acid residue. As the signal for the CH of quinic acid in the 3-position was shifted downfield in the spectrum of compound **5** (3.93 → 5.30 ppm) compared to that of compound **2**, **5** was identified as 3′,5′-dicaffeoylquinic acid (isochlorogenic acid). All of these phenylpropanoids had been previously reported in other lettuce cultivars (iceberg and romaine) (Hyodo et al., 1978; Winter and Herrmann, 1986), although their structures had never been confirmed by NMR. In addition, some controversy arose in a recent paper (Bennett et al., 1996) on the occurrence of isochlorogenic acid in lettuce. Bennett et al. (1996) did not find isochlorogenic acid in lettuce seedlings, while they did find chlorogenic, caffeoyltartaric, and dicaffeoyltartaric acids, in accordance with Winter and Herrmann (1986) and questioning the presence of isochlorogenic acid reported by other authors (Hyodo et al., 1978; Ke and Saltveit, 1989). These differences were probably due to the fact that isochlorogenic acid was detected by the latter authors in wounded or ethylene-exposed lettuce tissues, and this compound is induced by these stresses (see below). However, in Lollo Rosso isochlorogenic acid is present in quite large amounts, even in tissues that have not been subjected to wounding or ethylene stress.

Flavonols. The four flavonoids (**4**, **6**, **7**, and **9**) have a characteristic spectrum of quercetin 3-glycosides, and after acid hydrolysis all of them rendered quercetin and glucose with the exception of compound **6**, which yielded glucuronic acid. By paper electrophoresis at pH 4.4 compounds **4**, **6**, and **9** exhibited mobility toward the anode, showing that they were either acylated with dicarboxylic acids or glucuronides.

The UV study of compound **4** in methanol, and after the addition of the classical alkaline and chelating reagents (Mabry et al., 1970), showed that this was a quercetin derivative in which the hydroxyls in the 3- and 7-positions were blocked. When left in 2 N HCl for 24 h at room temperature, the naturally occurring glycoside was transformed in another with a shorter t_R in HPLC. Acid hydrolysis of the naturally occurring compound rendered quercetin, glucose, and malonic acid. The ^{1}H NMR spectrum clearly shows two anomeric protons of glucoses directly linked to hydroxyls of the quercetin (hydroxyls at 3 and 7) (Markham and Geiger, 1994) and the characteristic CH_2 response (singlet at 3.12 ppm) of the malonic acid residue linked to one of the sugars. When the ^{1}H NMR spectrum is compared with those of unacylated glycosides, the only difference is that there are two double doublets, which are shifted downfield in the acylated derivative. These signals are consistent with the 6_A and 6_B protons of the glucose, which are shifted downfield due to the acylation with malonic acid (Withopf et al., 1997). Enzymatic hydrolysis with β-D-glucosidase yielded compound **9**, supporting the assumption that the acylation was on the glucose in the 3-position. All of these data indicate that this is a compound similar to compound **9** but with an additional glucose linked in the hydroxyl group at position 7. Thus, compound **4** is quercetin 3-(6-malonylglucoside)-7-glucoside, a new naturally ocurring flavonoid (Figure 2).

Compound **6** showed a UV spectrum similar to that of a quercetin 3-glycoside. After acid hydrolysis, quercetin and glucuronic acid are observed. The FAB-MS (M^+ 480 and A + H 303) shows that this is a quercetin glucuronide. This cochromatographs with an authentic marker of quercetin 3-glucuronide.

Compound **7** showed a UV spectrum similar to that of a quercetin 3-glycoside. Acid hydrolysis yielded quercetin and glucose. This was identical by chromatographic comparisons with an authentic marker of quercetin 3-glucoside and confirmed its identity.

Compound **9** showed a UV−vis spectrum in methanol and after the addition of the classical shift reagents such as quercetin 3-glycoside (Mabry et al., 1970). Left in 2 N HCl at room temperature for 24 h, it was transformed into compound **7** (quercetin 3-glucoside). Compound **9** migrated toward the anode when analyzed by paper electrophoresis, suggesting that this was acylated with a dicarboxylic acid. After acid hydrolysis, quercetin, glucose, and malonic acid were detected. The ^{1}H NMR spectrum shows that this is a quercetin 3-glucoside and confirms the presence of malonic acid linked to the glucose residue in the 6-position (protons 6_A and 6_B of glucose shifted downfield to 4.21 and 4.03 ppm). This is also confirmed by FAB-MS in which a molecular ion at m/z 550 is observed corresponding to quercetin monoglucoside acylated with malonic acid. In addition, fragments consistent with the sequential loss of the malonyl residue (m/z 465) and the glucosyl residue (m/z 303) were also observed. ^{13}C NMR analysis clearly confirms that the malonyl residue is linked to the hydroxyl at the 6-position of the glucose as revealed by a downfield shift of 4 ppm for the C-6 of the glucose (60.0 → 63.6) and, therefore, this is quercetin 3-(6-malonylglucoside).

Anthocyanin. Compound **8** showed a UV−vis spectrum similar to that of a cyanidin derivative with a maximum at around 518 nm. Acid hydrolysis showed the presence of glucose and malonic acid. FAB-MS

Table 1. Mean (± Standard Deviations) of Total Phenolic Acids and Flavonols in White, Green, and Red Tissues of Lollo Rosso Lettuce Initially after Cutting and after 7 or 14 Days at 5 °C

	phenolic acids (μg g^{-1} fresh wt)	flavonoids (μg g^{-1} fresh wt)
white tissue		
initial	213 ± 60	43 ± 9
7 days	310 ± 50	34 ± 4
14 days	448 ± 89	55 ± 5
green tissue		
initial	570 ± 54	244 ± 16
7 days	540 ± 47	245 ± 20
14 days	588 ± 50	262 ± 38
red tissue		
initial	1696 ± 166	1384 ± 197
7 days	1756 ± 118	1686 ± 15
14 days	1418 ± 135	1340 ± 89

analysis of the isolated pigment confirmed that this was a cyanidin derivative, which contained, in addition, one glucosyl and one malonyl residue (536 M$^+$; 449 M − malonyl; 287 M − malonyl − glucose). This coincided by HPLC chromatography with an authentic marker of cyanidin 3-(6-malonylglucoside) previously isolated and identified from red onion (Ferreres et al., 1996).

Changes in Phenolics with Minimal Processing and Storage. The total soluble phenolic acid derivatives of minimally processed red lettuce midribs (white tissue) increased after 7 and 14 days of cold storage (Table 1). This is mainly due to the increase in isochlorogenic acid, which is the main caffeic acid derivative in this cultivar, and the amount increased 2-fold after 14 days of storage. Chlorogenic and caffeoyltartaric acids also increased after wounding with storage, while dicaffeoyltartaric acid remained quite constant (Figure 3). These results are similar to those previously reported for midribs of other lettuce cultivars (iceberg, romaine, and butter leaf) (Tomás-Barberán et al., 1997a,b). When green and red tissues were analyzed, this clear increase in the total phenolic acid content was not observed (Table 1). These tissues are very rich in this type of metabolites, and the wound response to induce the biosynthesis of these substances is not so relevant. However, isochlorogenic acid significantly ($p \leq 0.05$) increased in both tissues, although it remained constant in red tissue after 7 days. The content of chlorogenic acid in green and red tissues tremendously decreased (57.8 and 64.6%, respectively) from the initial values during storage. Chlorogenic acid has been described *in vitro* as one of the best PPO substrates (Janovitz-Klapp et al., 1990), and this could explain this decrease.

Lollo Rosso is much richer in phenolic acid derivatives than other previously studied cultivars. Thus, Winter and Herrmann (1986) reported between 65 and 270 μg g^{-1} of fresh weight of phenolic acids in whole lettuce, without differentiation between white and green tissues. However, the phenolic acid contents in midribs of romaine, iceberg, and butter leaf lettuce were much smaller (10−45 μg g^{-1} of fresh weight) (Tomás-Barberán et al., 1997b). In these tissues, wounding induced increases in phenolic acid content of 3-fold at 5 °C and 6-fold at 10 °C after 3 days storage. It seems that the wound-induced biosynthesis of phenylpropanoids in lettuce is much smaller in those tissues which are richer in preexisting phenolics, as is the case of Lollo Rosso. On the contrary, for tissues that are very poor in phenolics, as is the case of iceberg lettuce stems, the wound response induces an increase in phenolics which

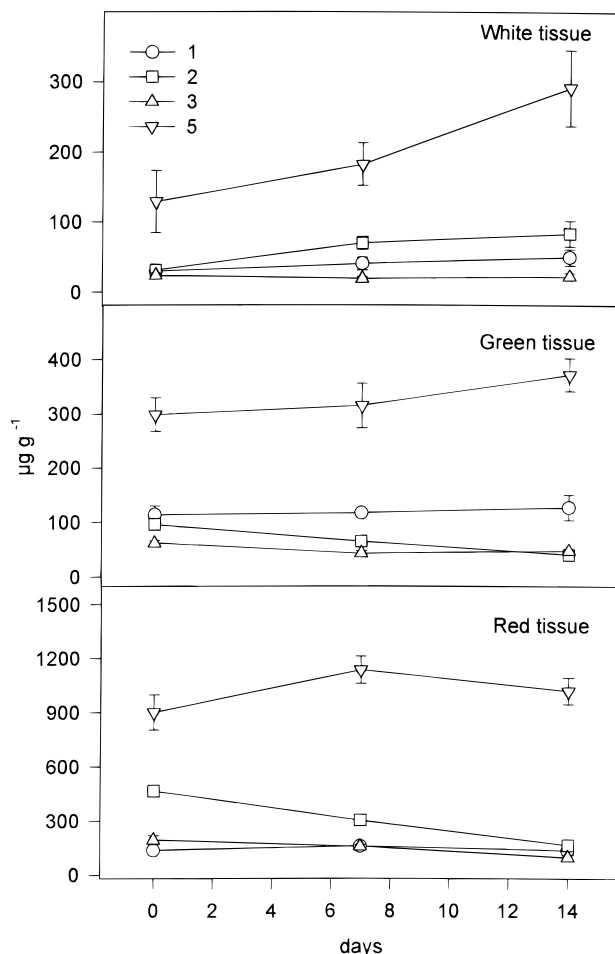

Figure 3. Changes in phenolic acids in white, green, and red tissues of Lollo Rosso lettuce kept at 5 °C for 7 and 14 days.

is 10-fold (from 2 to 20 μg g^{-1} of fresh weight) (Tomás-Barberán et al., 1997a).

A significant difference ($p \leq 0.05$) on the total flavonoid content was observed between tissues. While white tissue showed the smallest amount, the content of flavonoids in the red tissue was the highest (Table 1). These differences were expected due to tissue characteristics. Only one flavonoid, quercetin 3-(6-malonylglucoside) (**9**), was detected in the white tissue (Figure 3). From the green tissue, two flavonoids were identified as quercetin 3-glucoside and quercetin 3-(6-malonylglucoside), and they were very stable during the storage period. On the other hand, red tissue showed a very high flavonoid content in comparison with the other tissues and four flavonoids were isolated. Quercetin 3-(6-malonylglucoside) (**9**) increased up to 7 days to decrease at the end of the studied period. These fluctuations could be due to the hydrolysis of the malonyl group, since quercetin 3-glucoside (**7**) increased its content during the cold storage and no changes were observed for flavonoids **4** and **6** (Figure 4). The high concentration of flavonoids in Lollo Rosso has recently been reported (450−900 μg of quercetin g^{-1} of fresh weight) (Crozier et al., 1997), while other lettuce types had smaller amounts ranging between 10 and 90 μg g^{-1} of fresh weight. Previous papers indicate that lettuce had up to 250 mg of flavonoids g^{-1} of fresh tissue (Wöldecke and Herrmann, 1974).

Cyanindin 3-malonylglucoside was isolated and identified as the main anthocyanin pigment in red lettuce tissues. This agrees with previous work (Yamaguchi et al., 1996). The initial anthocyanin content in white

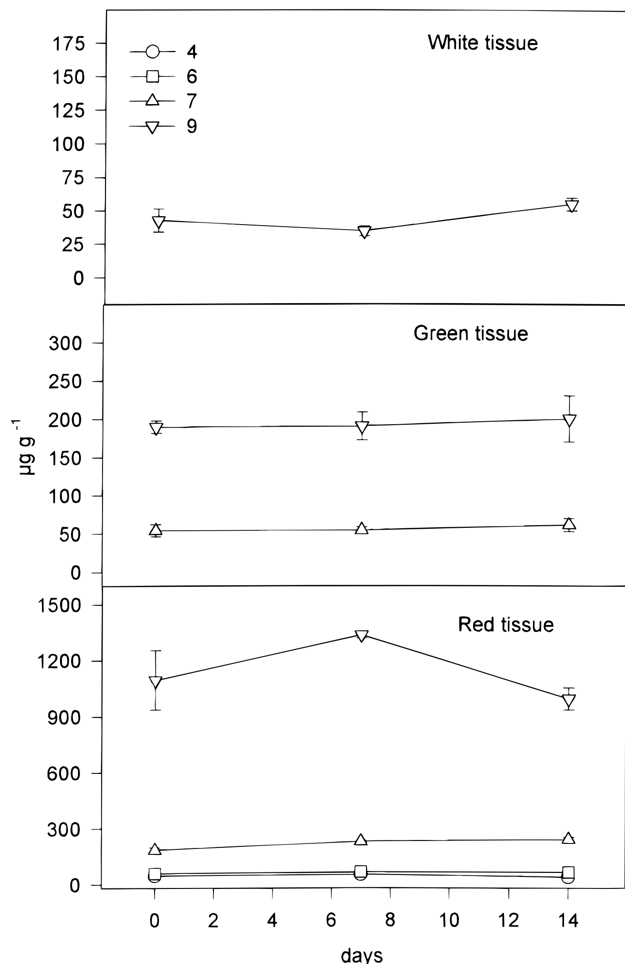

Figure 4. Changes in flavonoids in white, green, and red tissues of Lollo Rosso lettuce kept at 5 °C for 7 and 14 days.

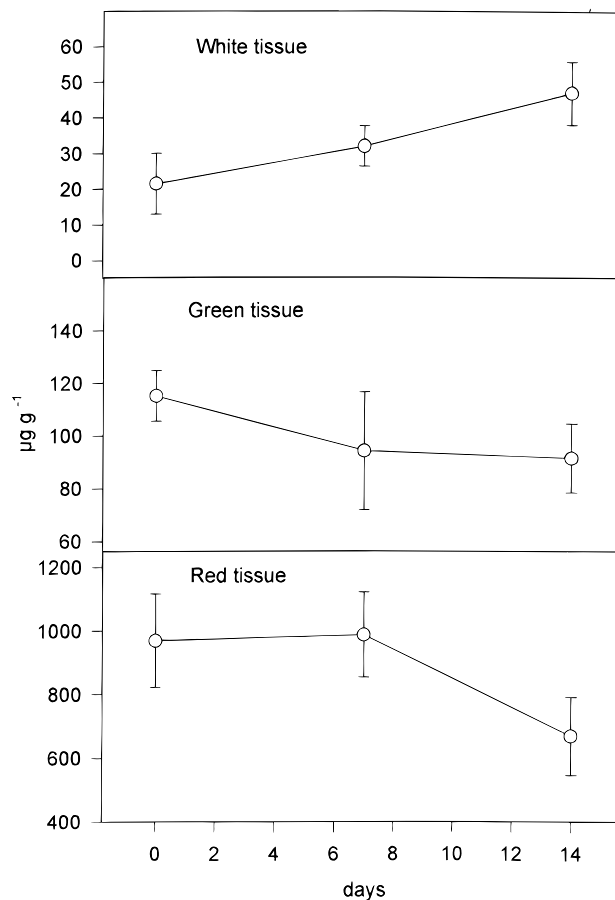

Figure 5. Changes in cyanidin 3-malonylglucoside in white, green, and red tissues of Lollo Rosso lettuce kept at 5 °C for 7 and 14 days.

tissue was very small (22 μg g^{-1}), but a significant ($p \leq 0.05$) increase was observed during cold storage (47 μg g^{-1}) (Figure 5). This increase with cold storage has been previously reported in maize seedlings (Christie et al., 1994), bush blueberry (Kalt and McDonald, 1996), and pomegranate (Holcroft et al., 1997). On the contrary, green and red tissue decreased their anthocyanin content from the initial values, immediately after cutting, to the end of the storage period (20.5 and 31.1%, respectively). The tendency in green tissue was an initial decrease during the first 7 days, and no changes were observed during the second week of storage. In red tissue the stability was the opposite, with no significant degradation during the first week and then a decrease during the second week of storage (Figure 5). The decrease in cyanidin 3-malonylglucoside was not due to a hydrolysis of the labile malonyl residue to render cyanidin 3-glucoside, since this pigment was not detected in significant amounts in the HPLC chromatograms.

As a conclusion, although midrib showed brown discoloration and, therefore, phenolics should be decreasing as PPO substrates, wounding induced phenolic metabolism and increased phenolic content throughout the storage. Phenolic acids and the anthocyanin cyanidin 3- malonylglucoside increased in midrib during cold storage, while flavonoids did not change. Finally, the total content of phenolic acids and flavonoids in both green and red tissues was maintained throughout the storage, but the anthocyanin concentration decreased.

These results show that the level of antioxidant and free-radical-scavenging flavonoids is maintained during the cold storage in air of minimally processed red lettuce. However, the pigment content of the red and green tissues decreased during the storage period and the phenolic compounds increased in the midrib, leading to browning; therefore, the visual quality of this product was reduced. These changes in phenolic metabolism should also be tested on minimally processed lettuce stored under modified atmospheres with high CO_2 and low O_2 levels, since under these conditions the visual quality of lettuce is maintained (López-Gálvez et al., 1996).

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